



# *The Journal of Laboratory and Clinical Medicine*

WARREN T. VAUGHAN, M.D., Editor  
201 W. Franklin St.  
Richmond, Va.

## ASSOCIATE EDITORS

### *Pharmacology*

DENNIS E. JACKSON, M.D.  
University of Cincinnati, Cincinnati

### *Tuberculosis*

GERALD B. WEBB, M.D.  
Cragmor Sanatorium, Colorado Springs

### *Clinical Pathology*

W. C. MACCARTHY, M.D.  
Mayo Clinic, Rochester, Minn.

T. B. MAGATH, M.D.  
Mayo Clinic, Rochester, Minn.

### *Biochemistry*

VICTOR C. MYERS, PH.D.  
Western Reserve University, Cleveland

### *Experimental Medicine*

RUSSELL L. HADEN, M.D.  
Cleveland Clinic, Cleveland

### *Immunology*

JOHN A. KOLMER, M.D.  
Temple University, Philadelphia

ROBERT A. KILDUFFE, M.D.  
Atlantic City, N. J.

### *Internal Medicine*

GEORGE HERRMANN, M.D.  
University of Texas, Galveston

### *Bacteriology*

M. H. SOULE, Sc.D.  
University of Michigan, Ann Arbor

### *Metabolism*

CLIFFORD J. BARBORKA, M.D.  
Northwestern University, Chicago

### *Surgery*

DEAN LEWIS, M.D.  
Johns Hopkins University, Baltimore

---

VOLUME 24  
OCTOBER, 1938—SEPTEMBER, 1939

---

ST. LOUIS  
THE C. V. MOSBY COMPANY  
1939



Copyright, 1939, By The C. V. Mosby Company  
*(All rights reserved)*

Printed in the  
United States of America

# *The Journal of Laboratory and Clinical Medicine*

---

VOL. 24

OCTOBER 1938

No. 1

---

## *CLINICAL AND EXPERIMENTAL*

---

### THE DIFFERENTIAL SERUM VANADATE SEDIMENTATION REACTION\*

---

H. B. HUNT, M.B., M.R.C.S. AND D. L. WOODHOUSE, PH.D., A.I.C.,  
BIRMINGHAM, ENGLAND

---

OUR knowledge concerning the physicochemical colloidal protein system existing in blood serum is very recent and imperfect. From clinical and pathologic standpoints some appreciation of its complexity and significance is now being afforded by researches, especially in conditions where normal equilibria appear to be definitely modified. Contributions from these aspects have been made by Coke (1937) who has applied the sodium vanadate reagent, which was introduced by Bendien (1931) as the precipitant in his diagnostic test for malignancy, employing the three phase method of serum flocculation evolved by Cronin Lowe<sup>4</sup> (1933) as a means of scrutinizing the colloidal system of the serum.

One of the present authors (D. L. W.) has extensively employed the modified vanadate sedimentation reaction with a view to estimating its value as a diagnostic test for carcinoma (Jones and Woodhouse, 1936) and, although results showed that it can not be regarded as a specific test for cancer, these and subsequent studies confirm the suggestion of Cronin Lowe<sup>4</sup> (1933-34) and others, that when properly interpreted it affords useful information for prognosis and for control of therapeutic measures in malignant disease.

A similar conclusion is drawn by Coke<sup>5</sup> (1937) concerning the application of the modified vanadate reaction to arthritis and to asthma, in which he claims it to be of great practical value. To quote this investigator: "It provides (1)

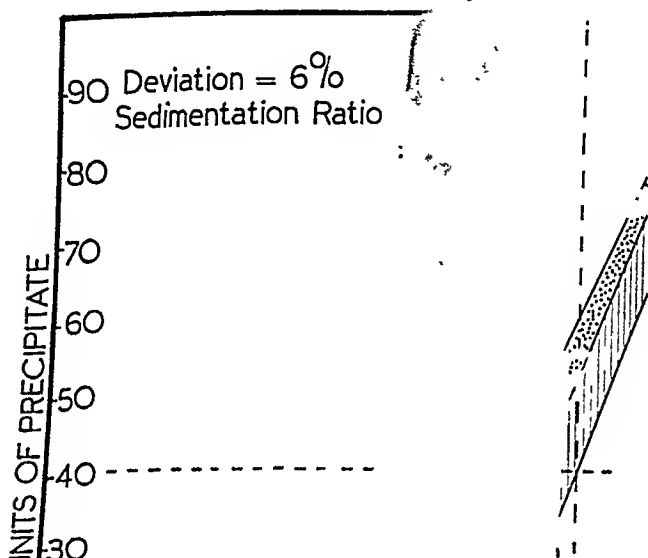
---

\*From the Asthma Clinic and the Cancer Research Laboratory, General Hospital, Birmingham.

Received for publication January 8 1938

a reading comparable in every way to the erythrocyte sedimentation rate but . . . which is a truer indication of the actual clinical state. (2) Evidence of the state of the infectious processes present and the patient's response to these. (3) Evidence as to the 'general toxicity' and 'general resistance' of the patient. (4) The possibility of watching the progress of cases by serial examination and, in certain cases, controlling the therapeutic methods exhibited."

Since one of us (H. B. H.) was particularly interested in asthma, it was deemed of value in view of these assertions to observe the vanadate sedimentation values in asthmatics and to compare with the red blood sedimentation re-



For each serum three series of precipitation tests, A, B, and C, are set out, using for each series ten portions of 0.2 cc. of clear, nonhemolyzed serum, which has been diluted with an equal volume of distilled water, 1 cc. of the ten vanadate reagents (containing 21, 22, 23, to 30 cc. of N/10 sodium vanadate, each made up to a total volume of 200 cc. with N/10 acetic acid) is added to the tubes in order. These are numbered in accordance with the number of cubic centimeters of N/10 sodium vanadate in 200 cc. of the reagent, viz., 21, 22, 23, etc.

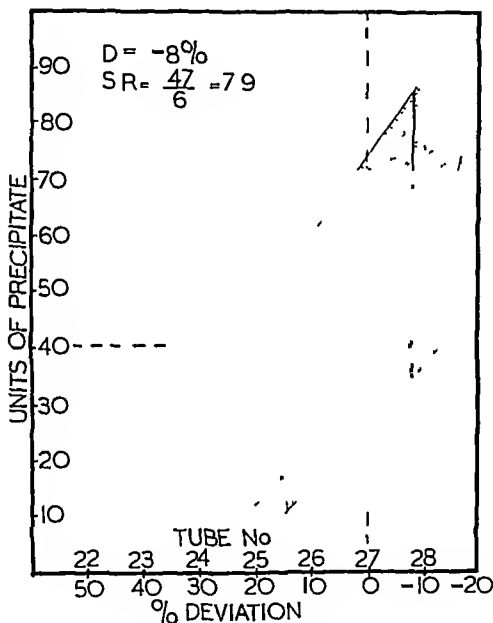


Chart 2 — Asthma Type I chart

The A series is set up by using the diluted serum directly, the B series employs the same serum heated for thirty minutes at  $56^{\circ}\text{C}$ , and for the C series the serum has been treated by extraction with ether. The precipitation with the A series for healthy sera becomes definite in tubes 26 and 27, and increases with the concentration and pH of the vanadate reagent added. In pathologic cases the precipitation zone is shifted to the left or more rarely to the right. In the B series the amount of precipitation for any specified reagent is usually distinctly less than in the corresponding A series and is definitely greater in the C series.

In assessing the results for any individual test, three factors must be considered

*the Differential Sedimentation Ratio.*—After estimating the amount of precipitate produced in the various tubes in suitable units, by means of the interferometer or other accurate method, the graph of the degree of flocculation is drawn for each of the three series (see charts). The sedimentation ratio may then be calculated for the specimen at any desired point. This is the difference in flocculation at that point between the A and C series compared with the difference in flocculation between the A and B series as expressed by the ratio

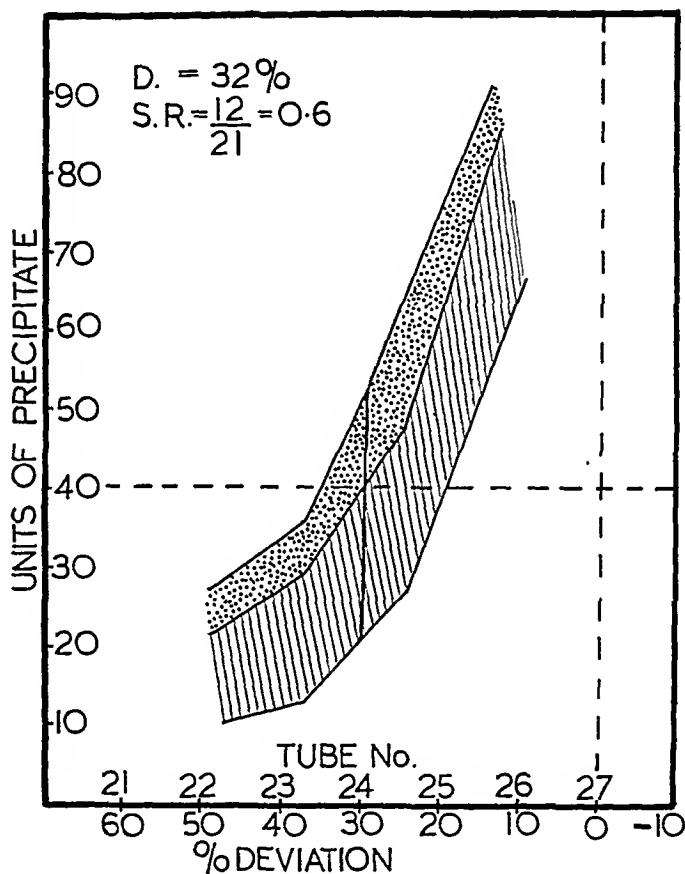
$$\frac{C - A}{A - B}$$


Chart 3.—Asthma "Type II" chart.

Since the most sensitive part of the precipitation zone was found to lie in the position where the precipitate from the untreated serum registered 40 units on the Zeiss interferometer when dissolved under standard conditions, the ratio is taken at this position for comparing sera (i.e.,  $\frac{C' - A'}{A' - B'}$ , Chart 1). For normal sera this occurs in the majority of cases at tube 27.

2. *Sedimentation Deviation.*—An alteration from the "normal" so that it occurs at tube 26 (see charts) is designated a sedimentation deviation of 10 per cent, if at tube 25, 20 per cent deviation, and if at tube 28, -10 per cent deviation; i.e., one tube represents 10 per cent. The deviation may be sometimes as much as +100 per cent or -25 per cent in pathologic cases.

According to the original Bendien hypothesis, a marked positive deviation indicates malignancy, but large deviations were found to be exhibited in many pathologic conditions.

Cronin Lowe<sup>1</sup> suggested that the ratio  $\frac{C - A}{A - B}$  at the 40 unit precipitation point was a more reliable index. This is the basis of his modified vanadate reaction, the ratio being greater than unity with malignant sera. As previously mentioned, this has been found to be not entirely reliable, but the fact has been confirmed that the ratio often becomes more normal in type when the cancer is

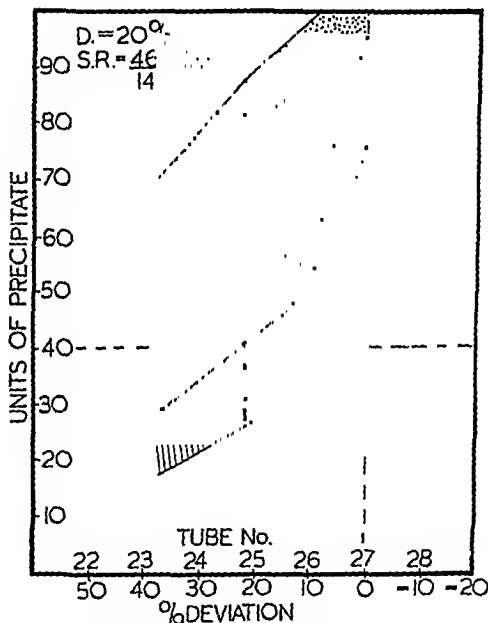


Chart 4.—Malignant case (No. 562, Table V).

responding satisfactorily to therapeutic measures. Coke believes that the whole picture expressed by the three curves (see Charts 1 to 7) should be studied in considering any case.

3. *Dotted and Striped Fields.*—The area between the A and C curves (shown by dots and called the dotted field) is believed to indicate the degree of intoxication, and the area between the A and B curves (shown by stripes and termed the striped field) to be a measure of the resources of the patient in combating the infection.

Coke also claims that by means of the differential sedimentation reaction, it is possible to divide asthmatics into three groups which correspond to the

TABLE I  
CLINICAL FEATURES AND LABORATORY FINDINGS

LAB. NO.	AGE	SEX	FAMILY HISTORY	OTHER ALLERGIC DISEASE	SEVERITY OF ASTHMA	CHRONIC BRONCHITIS	TREATMENT	EOSINOPHILE COUNT PER CENT	ERYTHROCYTE SEDIMENTATION RATE IN MM.	PERCENT-AGE DEVIATION	SEDIMENTATION RATIO
<i>A</i>											
864	14	M	Pos.	H.F.	Moderate		Nil	7.5	28	3	9.6/12.0 = 0.8
867	25	F	Pos.	H.F.	Severe	Present	Nil	3.5	3	2	4.0/10.8 = 0.4
876	13	M	Pos.	R.	Moderate	Present	Nil	8.0	25	-4	10.0/13.0 = 0.8
877	35	F	Neg.	R.	Mild	Present	Nil	7.0	6	-4	11.0/14.0 = 0.76
880	26	M	Pos.	H.F.	Moderate		Nil	3.0	2	-2	9.0/14.0 = 0.6
883	40	F	Neg.		Severe	Present	Peptone	5.0	13	2	8.0/14.0 = 0.6
884	27	F	Pos.	R.U.	Moderate		Nil	5.0	7	-2	6.0/8.8 = 0.7
887	20	M	Pos.	H.F., R.	Severe		Nil	8.5	5	-2	10.0/15.0 = 0.7
899	37	F	Neg.	H.F.	Moderate	Present	Pollen vaccine	0.0	4	0	12.0/16.0 = 0.8
906	32	F	Pos.	H.F.	Moderate		Nil	4.0	10	8	2.8/16.0 = 0.25
912	67	F	Neg.	R.	Moderate	Present	Bact. vaccine	1.0	5	0	8.0/10.4 = 0.8
926	38	M	Neg.	R.	Severe	Present	Peptone		3	3	6.0/12.0 = 0.5
<i>B</i>											
861	56	F	Pos.	H.F., R.	Moderate		Nil	1.5	11	0	32.0/44 = 7.3
901	31	F	Neg.		Mild		Torantil	6.0	9	-2	10.0/64 = 1.5
902	50	F	Pos.		Moderate		Bact. vaccine	8.0	8	0	18.8/64 = 3.0
907	14	M	Neg.	R.	Mild		Nil	6.0	3	-3	16.8/6.8 = 2.5
911	28	F	Pos.	H.F.	Severe		Peptone	3.5	4	6	10.0/6.4 = 1.7
925	37	M	Neg.	H.F.	Severe		Peptone		6	4	10.0/9.0 = 1.1

H. F. = Hay fever. R. = Rhinorrhea. U. = Urticaria.

TABLE II  
CLINICAL FEATURES AND LABORATORY FINDINGS

LAB NO	AGE	SEX	FAMILY HISTORY	OTHER ALLERGIC DISEASE	SEVERITY OF ASTHMA	CHRONIC BRONCHITIS	TREATMENT	EOSINOPHIL COUNT PERCENT	ERYTHROCYTE SEDIMENTATION RATE IN MM	DIFFERENTIAL COUNT	SEDIMENTATION RATIO
<i>A</i>											
S36	40	F	Pos	HF	Moderate		Nil	40	2	32	30/120 = 0.25
S37	24	F	Neg	Ecz	Mild		Nil	70	1	20	120/160 = 0.75
S38	60	M	Neg		Severe	Present	Torantul	30	4	40	10/68 = 0.6
S42	51	F	Pos	HF	Moderate	Present	Nil		2	20	160/200 = 0.80
S43	36	F	Neg		Mild		Nil	35	7	16	72/128 = 0.60
S44	36	F	Pos		Moderate		Bact vaccine	80	2	10	20/20 = 1.0
S56	63	M	Neg	HF	Moderate	Present	Bee venom	60	2	10	20/40 = 0.25
S37	64	F	Neg		Severe	Present	Nil	40	8	24	16/12 = 1.0
S59	58	F	Pos	HF	Severe	Present	Bact vaccine		27	20	140/160 = 0.90
S67	47	F	Pos	HF	Moderate		Bact vaccine	80	6	22	120/120 = 1.0
S67	47	F	Pos	HF	Mild		Bact vaccine	85	7	22	108/160 = 0.63
<i>B</i>											
S39	19	F	Pos		Moderate	Present	Torantul	25	2	16	220/20 = 11.0
S40	42	F	Pos	HF	Mild		Bact vaccine	10	5	32	88/40 = 4.7
S41	18	M	Neg	HF	Mild		Pollen vaccine	20		17	108/40 = 2.7
S58	36	F	Pos	HF	Moderate		Pollen vaccine	75	7	24	208/40 = 5.2
S60	51	F	Pos		Moderate		Nil	80	3	26	180/128 = 1.4
S62	52	F	Pos	HF	Moderate	Present	Bee venom	140	10	10	220/40 = 5.5
S65	29	M	Pos	HF, R	Moderate		Nil	160	12	14	60/44 = 1.4

Ecz = Dermatitis



clinical classification so commonly employed, viz., pure allergic, infective, and mixed types. The features of these types from the point of view of the differential sedimentation reaction, are summarized below:

Type I. Pure Allergic Type (Chart 2)

- Differential ratio generally above 3.
- Percentage deviation commonly negative.
- Dotted field enlarged, striped field reduced.

Type II. Bronchial Infective Type (Chart 3)

- Differential ratio usually below 1.
- Percentage deviation positive and often high, e.g., 20 per cent.
- Dotted field normal, striped field increased.

Type III. Mixed Type

- Differential ratio commonly between 1 and 2.
- Percentage deviation variable, e.g., 30 per cent to -10 per cent.
- Dotted and striped fields enlarged.

For the red blood cell sedimentation determinations described in this paper the micromethod employed in the Midhurst Sanatorium (Vide Midhurst Report, 1933-34) was used. It lends itself to routine clinical estimations, requiring only a small volume of blood, and is simple in procedure. The sedimentation tubes are of thick glass, 15 cm. long, 1.13 mm. bore, and are graduated in millimeters. For each test 0.4 c.c. of the same sample of venous blood as used for the vanadate reaction is drawn into a 1 c.c. syringe containing 0.1 c.c. of sterile 3.8 per cent sodium citrate solution, thoroughly mixed in a capsule, sucked into the sedimentation tube, adjusted so that the column is 100 mm. in length, and set up in the rack at room temperature.

After one hour the clear zone is observed, its depth in millimeters being the figures recorded as the sedimentation rate. From the citrated blood a film was prepared from which the eosinophile count was made after staining with Jenner's stain.

#### MATERIAL AND RESULTS

The series of cases presented in this paper are made up of a group of 50 patients suffering from bronchial asthma, a group of 25 patients suffering from various other nonmalignant conditions uncomplicated by asthma, and a group of 50 patients with definite and moderately advanced malignancy.

The last two groups are taken from the sera examined since the initial communication by one of the present writers (D. L. W.) on the modified vanadate reaction (Jones and Woodhouse) and comprise additional material, since the full charting method was not employed in the previous investigation.

The results of all the analyses were charted according to the method outlined above, and the deviations and ratios so calculated are listed in Tables I to V. Differential sedimentation curves of some typical cases are also included in Charts 4 to 7.

It has been satisfactorily demonstrated by other workers, e.g., Cuttler (1932), Kessler (1936), and Reichel (1936), that there is no specific correlation between neoplasia and the rate of blood corpuscle sedimentation, though there is an increased rate of fall in many cases of malignancy probably due to tissue destruction. Therefore, no sedimentation figures are given for these cases included in Tables IV and V.

## DISCUSSION

Of the 50 asthmatics examined in the present investigation 19 had ratios of the type formerly called "malignant," although the probability of undetected cancer among this group was relatively small considering the ages of the patients, etc.

The series of malignant cases in this investigation yielded a very high proportion of ratios "positive for cancer," viz, 86 per cent (Table IV). Possibly this is due in part to the fact that the patients in question were all in a moder-

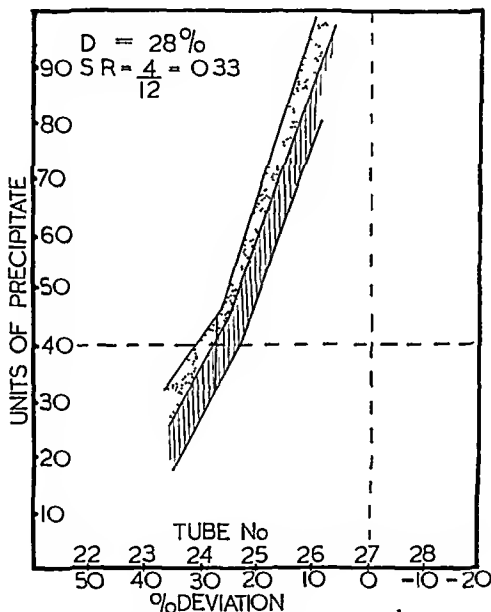


Chart 5—Nonmalignant case (No. 624 Table IV)

ately advanced, though not terminal, state. The series of nonmalignant cases also has yielded a fairly satisfactory return in that 76 per cent of "negative" returns are recorded (Table V).

In reviewing the clinical data of the asthmatic patients the following points were noted: age and sex, family history of allergy, presence of any other allergic disease, any acute pathologic process in action, and any treatment in course. In 20 cases the tests were carried out prior to any specific treatment.

Tables I, II, and III contain results and data relevant to the asthmatic patients. They have been grouped into three divisions from a consideration of

TABLE III  
CLINICAL FEATURES AND LABORATORY FINDINGS

LAB. NO.	AGE	SEX	FAMILY HISTORY	OTHER ALLERGIC DISEASE	SEVERITY OF ASTHMA	CHRONIC BRONCHITIS	TREATMENT	EOSINOPHILIC COUNT PER CENT	ERYTHROCYTE SEDIMENTATION RATE IN MM.	PERCENT AGT DEVIATION	SEDIMENTATION RATIO
<i>A</i>											
866	20	F	Neg.		Severe	Chronic	Bact. vaccine	9.0	3	-7	10.0/12.0 = 0.83
886	36	F	Neg.		Moderate		Nil	2.0	2	-10	12.0/10.4 = 1.2
890	21	F	Neg.		Mild		Bact. vaccine	8.5	5	-15	5.2/13.2 = 0.4
895	33	M	Neg.		Mild	Present	Peptone	3.0	4	-17	6.1/ 9.2 = 0.7
897	10	F	Neg.		Moderate	Present	Nil	11.0	4	-7	6.0/10.0 = 0.6
900	11	F	Neg.	H.F., R.	Severe		Nil	2.0	4	-14	6.0/16.0 = 0.4
904	39	F	Pos.	H.F.	Moderate		Pollen vaccine	6.0	2	-11	6.8/ 9.2 = 0.7
915	33	F	Neg.		Severe		Bact. vaccine	6.0	3	-6	16.8/ 18.8 = 0.9
<i>B</i>											
868	23	F	Neg.		Moderate		Bact. vaccine	5.0	2	-18	27.0/ 1.2 = 22.7
871	9	M	Neg.		Moderate		Nil	5.0	2	-15	18.0/ 6.0 = 3.0
875	22	M	Neg.	R.	Moderate	Present	Nil	5.0	12	-15	12.8/ 8.0 = 1.6
885	33	M	Neg.	H.F.	Moderate	Present	Nil		1	-18	27.2/ 6.0 = 4.5
891	32	M	Pos.	H.F.	Moderate		Nil	9.0	5	-7	22.0/ 8.8 = 2.5
896	31	F	Pos.	H.F.	Mild		Pollen vaccine	8.0	2	-25	15.0/ 5.0 = 3.0
908	20	F		H.F.	Mild		Peptone	11.0	6	-13	16.0/ 4.2 = 3.3

TABLE IV  
NONMALIGNANT CASES (PATHOLOGIC)

LAB NO	PATHOLOGIC CONDITION	SEDIMENTATION DEVIATION PER CENT	DIFFERENTIAL SEDIMENTATION RATIO
501	Empyema	90	14/29 = 0.5
509	Gastric ulcer	50	4/28 = 0.14
577	Menopausal hemorrhage	24	4.8/8 = 0.6
554	? tuberculosis	75	4/16 = 0.25
590	Veneral disease (? cured)	20	6/8 = 0.75
601	Ulcer pilate (histologically non malignant)	15	4/28 = 3.3
602	For repair uterus	80	4.8/20 = 0.28
611	Tuberculosis pilate (history non malignant)	50	10/20 = 0.5
613	Lead poisoning	78	25/36 = 0.7
623	Rheumatoid arthritis	55	2/28 = 0.07
624	Gastric ulcer	28	4/12 = 0.33
628	Jaundice (relieved)	50	12/18 = 0.7
629	Gastric ulcer	72	20/20 = 1.0
630	Colitis	16	28/30 = 0.9
641	Dysmenorrhea	25	4/36 = 0.11
644	Diabetes	50	12/26 = 0.46
646	Ovarian cyst	30	6/12 = 0.5
650	Gastric ulcer	32	12/14 = 0.8
651	Diabetes	15	20/20 = 1.0
500	Duodenal ulcer	25	48/24 = 2.0
501	Dysmenorrhea	4	46/20 = 2.3
518	Duodenal ulcer	26	56/28 = 2.0
507	Cholecystitis	20	72/14 = 5.2
513	Bronchopneumonia	80	14/14 = 3.5
634	Dysmenorrhea	18	72/28 = 1.1
600	Normal healthy	0	2.4/22 = 0.18
610	Normal healthy	12	20/24 = 0.8

the type of chart obtained. Table I contains those cases in which only slight deviations were evident, the greatest being 8 per cent and the lowest -4 per cent. In Table II the deviations are in every case 10 per cent or greater and all positive. In Table III the sera giving marked negative deviations are set out. In each case these groups have been subdivided into two sections, A and B, the first comprising those in which the sedimentation ratios were approximately equal to or less than unity, and the second containing those with ratios definitely greater than unity.

This method of presentation of results should, if the claims of Coke can be accepted as generally valid, distribute the cases into the clinical groups viz., allergic, infectious, and mixed. Employing this method of interpretation, it is obvious that the cases included in Table IIA and those in Table IIIB should in the main coincide respectively with the "bronchial infective" and "pure allergic" types of asthma. However, of the 12 cases in Table IIA, only 5 could, on clinical grounds, be found to possess any similarity to the "bronchial infective" type of asthma, and of the 7 cases in Table IIIB, only 3 showed any degree of clinical correspondence. Clinically there seemed to be no marked difference between those cases in the two groups just mentioned which failed to coincide and those in other groups, such as Tables IA and IB.

TABLE V  
MALIGNANT CASES

LAB. NO.	PATHOLOGIC CONDITION	SEDIMENTATION DEVIATION PER CENT	DIFFERENTIAL SEDIMENTATION RATIO
502	Carcinoma cervix	25	26/15 = 1.7
503	Carcinoma breast	18	44/12 = 3.7
505	Epithelioma penis	10	88/12 = 7.3
522	Carcinoma breast	30	30/12 = 1.9
526	Carcinoma thyroid	10	38/10 = 3.8
528	Carcinoma breast	25	46/16 = 2.9
530	Carcinoma stomach	90	15/12 = 1.25
540	Carcinoma bronchus with secondaries	90	24/16 = 1.5
546	Adenocarcinoma pelvis	100	12/10 = 1.2
548	Sarcoma femur	10	42/20 = 2.1
558	Carcinoma stomach	10	40/24 = 1.7
562	Carcinoma breast	20	46/14 = 3.3
566	Carcinoma stomach	90	32/19 = 2.2
578	Ostrogenic sarcoma	20	44/24 = 1.8
587	Spheroidal cell carcinoma breast	25	46/26 = 1.8
592	Carcinoma cervix	32	28/12 = 2.3
615	Carcinoma stomach	60	40/ 4 = 10.0
616	Carcinoma colon	80	24/12 = 2.0
622	Carcinoma stomach	50	40/ 4 = 10.0
627	Carcinoma lung	60	24/ 4 = 6.0
618	Carcinoma stomach	40	23/ 2 = 11.5
550	Recurrent carcinoma breast (glands)	30	4/16 = 0.25
551	Carcinoma cervix	20	10/30 = 0.33
647	Epithelioma vulva	36	4/24 = 0.17
626	Carcinoma cervix	40	33/40 = 0.8
515	Carcinoma breast	14	40/32 = 1.25
519	Carcinoma perineum	50	28/ 5.0 = 5.6
520	Carcinoma colon	70	16/12 = 1.3
521	Carcinoma breast	10	26/14 = 1.9
525	Epithelioma tongue	22	24/ 4 = 6.0
527	Epithelioma face	10	46/27 = 1.7
531	Epithelioma tongue	34	36/18 = 2.0
532	Epithelioma tongue (recurrent)	30	52/30 = 1.7
533	Carcinoma breast	10	76/11 = 4.8
534	Epithelioma floor of mouth	24	46/20 = 2.3
535	Carcinoma lung	55	30/ 2 = 15.0
542	Epithelioma tongue	18	60/12 = 5.0
543	Carcinoma breast	18	26/12 = 2.2
545	Carcinoma ovary	52	16/14 = 1.1
553	Epithelioma ear	40	36/28 = 7.3
563	Carcinoma breast	40	40/12 = 3.3
567	Epithelioma tongue	55	33/ 6.8 = 4.9
555	Epithelioma nipple	16	28/16 = 1.8
568	Epithelioma face	33	41/24 = 1.7
572	Carcinoma breast	30	65/ 6 = 10.9
604	Epithelioma face	25	46/46 = 1.0
625	Carcinoma cervix	50	32/12 = 2.7
559	Epithelioma tongue	10	28/40 = 0.7
561	Epithelioma lip	10	36/44 = 0.8
623	Carcinoma cervix	35	16/24 = 0.7

On the contrary an uncomplicated case of hay asthma (880) examined when symptoms were present did not show the typical serologic findings which according to Coke characterize the "pure allergic" group. In another instance a patient (883), who on clinical grounds would be placed in the "bronchial infective" group, gave a normal differential sedimentation reaction.

But although we were not able to distinguish by means of the sedimentation data between the clinical types as definitely as would be expected from the description by Coke, yet the cases studied presented distinguishing features as a whole. Thus among the 75 pathologic cases not one instance of a nega

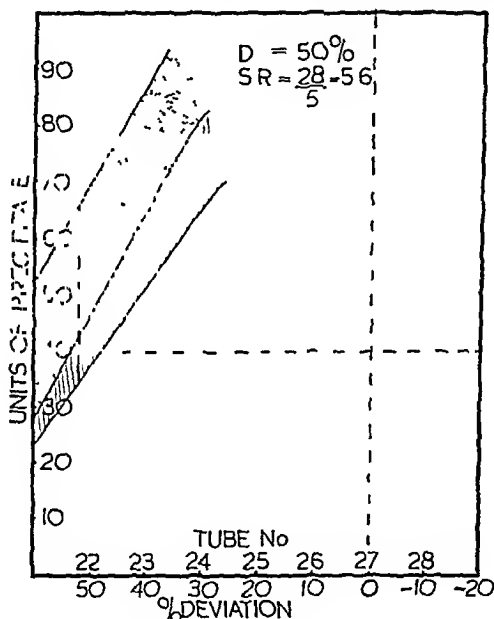


Chart 6—Malignant case (No 519 Table V)

tive serum sedimentation deviation was encountered while among the asthmatics no less than 15 occurred with definite negative deviations, and 11 of these to the extent of 10 per cent or more.

It did not seem possible, however, to correlate the individual results of the differential sedimentation tests with the clinical picture in a manner which was thoroughly convincing.

Also examination of Tables I, II, and III shows that it is difficult to correlate the blood sedimentation rate with the percentage deviation. Thus definite acute infections (864, 876, Table I) with fast sedimentation rates had normal deviations while normal sedimentation rates (Table II) were accompanied by

high deviations. Again in Table III, where high negative deviations are grouped, there is no evidence of very slow blood sedimentations.

Since negative deviations seem to be so characteristic of asthma, it was considered possible that a relationship might exist between these and the eosinophile count. The eosinophile counts in Tables I, II, and III were not in all cases made from the same samples of blood which were employed for the blood sedimentation rate and differential sedimentation reaction estimations. Usually, however, they were prepared within a few weeks of these tests.

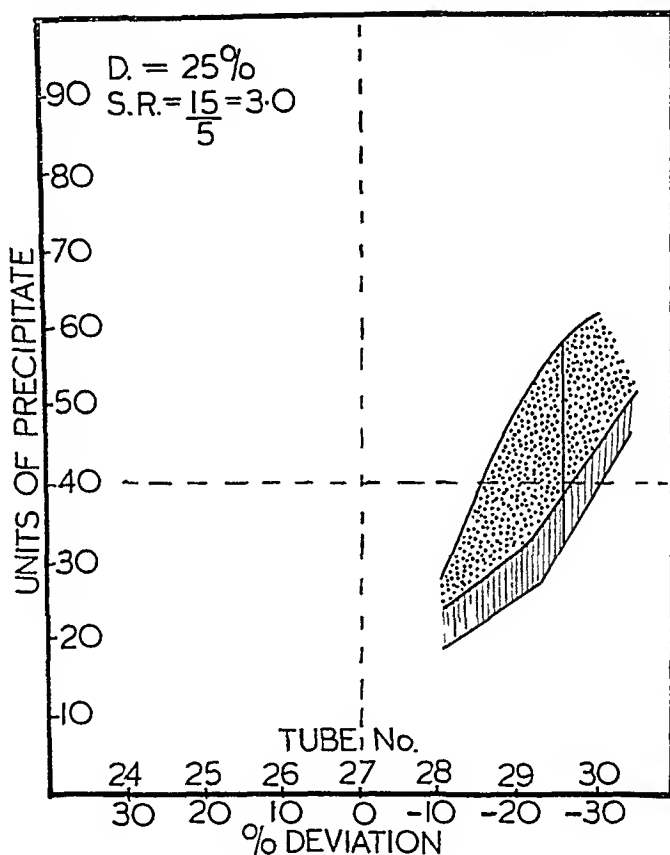


Chart 7.—Asthmatic case (No. 896, Table III B).

The average eosinophile count for the cases with negative deviations (Table III) is 6.5, and for those cases with positive deviations (Table II) is 6.7. In the present circumstances so small a difference appears of no significance. It is thus not possible to correlate the type of deviation with the eosinophile count.

#### SUMMARY AND CONCLUSION

The differential serum sedimentation figures have been determined for a group of 50 asthmatic patients, a group of 50 malignant patients, and 25 patients suffering from nonmalignant diseases, and the corresponding charts drawn for the values. In the asthmatic series determinations of the red blood corpuscle sedimentation rates and the eosinophile counts were also made.

The differential serum sedimentation tests did not appear to give definite information from the point of view of clinical classification of the asthmatic patients. About 30 per cent of these, however, showed a very definite negative deviation, a feature not observed in other groups.

It was not found possible to correlate the degree of eosinophilia with the sedimentation deviation or with the blood corpuscle sedimentation rate.

The vanadate sedimentation estimations have been carried out as a part of the inquiry into serological reactions especially in relation to malignant disease by one of the authors (D.L.W.) under the auspices of the British Empire Cancer Campaign Birmingham Section. For facilities in connection with the Asthma Clinic Patients our best thanks are due to Professor W. H. Wynn.

#### REFERENCES

- 1 Bendien, S. G. T. Specific Changes in Blood Serum, Transl. by A. Pinev, London, 1931.
- 2 Coke, H. Charterhouse Rheumatism Clinic Original Papers, The Differential Sedimentation Test 1-43, 1937.
- 3 Cuttler, J. W. Practical Application of Blood Sedimentation Test in General Medicine, *Am J M Sc* 183 643, 1932.
- 4 Lowe, E. C. Quantitative Modification of Bendien Reaction in Sero Diagnosis of Malignancy, *Brit M J* 1 407, 1923.
- 5 Lowe, E. C. Value of Serum Reactions in Radiotherapy of Cancer, *Brit J Radiol* 6 207, 1933.
- 6 Lowe, E. C. Serological Observations From Cases of Cancer Under Treatment, *J State Med* 42 444, 1934.
- 7 Jones, R. E., and Woodhouse, D. L. The Modified Vanadate Serum Malignancy Test, *Quart J Med* 5 141, 1936.
- 8 Kessler, R. Die Blutkörperchen-senkungsreaktion in der Differentialdiagnose maligner Tumoren, *Deutsch med Wchnschr* 62 258, 1936.
- 9 Reichel, H. (Vienna) Blutkörperchen-senkung bei malignen Tumoren, *Med Klin* 32 1769, 1936.

## TRICHOSTRONGYLUS COLUBRIFORMIS IN THE HUMAN APPENDIX\*

### REPORT OF A CASE IN LOUISIANA

JOHN R. SCHENKEN, M.D., AND E. S. MOSS, M.D., NEW ORLEANS, LA

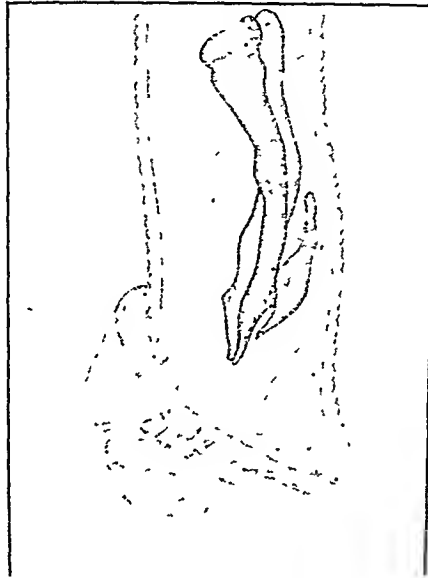
**A** NEMATODE was discovered in the appendiceal content of an 11 year old white female, native of Louisiana, whose appendix had been removed because of recurrent attacks of abdominal pain. Professor E. C. Faust, of the Tulane University School of Medicine, identified the parasite as an adult male *Trichostrongylus colubiformis*. Although members of the *Trichostrongylidae* are commonly present in the intestinal tract of sheep and goats in North America, no instance of human infection with this parasite has been previously reported in the Western hemisphere. Human parasitism by *T. colubiformis* has been observed in Egypt, India, Armenia, and Australia, particularly among individuals in sheep and goat raising localities. Although we do not believe that the parasite was responsible for the complaints of the patient, we feel that its discovery in the intestinal canal of a human being should be recorded.

\*From the Departments of Pathology and Bacteriology of the School of Medicine Louisiana State University and the State Charity Hospital of Louisiana New Orleans.  
Received for publication January 10 1938



## REPORT OF CASE

On August 15, 1937, P. S., an 11-year-old white female, experienced a sudden cramping abdominal pain which localized to the right of the umbilicus. She soon became nauseated and vomited several times. The administration of a mild laxative caused no alteration of the symptoms. The pain persisted as a dull discomfort with knifelike exacerbations, but never radiated or became localized in the right lower quadrant. She was admitted to the Charity Hospital on August 29. She had experienced headache, vertigo, and mild attacks of abdominal pain for about one year, which was thought to be caused by a chronic recurrent appendicitis. On admission the physical examination revealed a well-nourished and well developed ambulatory child, with a normal temperature, pulse rate, respiratory rate, and blood pressure. Except for slight tenderness and rigidity of the abdomen, about 5 cm. to the right of the umbilicus, the physical findings were normal. An appendectomy was performed on August 31. The surgeon saw no evidence of an acute inflammation or periappendicial adhesions. The



Since several of the standard textbooks of parasitology contain excellent descriptions of the *Trichostrongyloidea*, we are only submitting a drawing of the bursa of the specimen, showing the characteristic copulatory spicules.

#### DISCUSSION

According to Craig and Faust (1937) several hundred worms are necessary to provoke marked clinical manifestations. Our case showed no anemia or emaciation and undoubtedly was a light infection, possibly only a single worm being present. In view of the fact that two other parasitic species were discovered in the intestinal content, we were unable to hold the *Trichostrongylus* responsible for the mild chronic inflammation in the appendix. The patient's symptoms and signs suggested an appendiceal colic and, although the parasite may have been a factor in the production of spasm, the *Enterobius* alone could initiate these symptoms. No definite information could be obtained regarding the source of the infection. The patient lives in the city and her only contacts with ruminants were those at the municipal park and an occasional visit in the country where very few sheep or goats are raised.

#### SUMMARY

1. An instance of *Trichostrongylus colubriformis* in the human appendix is reported.
2. No etiologic relationship between the parasite and the appendiceal inflammation can be established.

#### REFERENCES

1. Craig, C. F., and Faust, E. C.: *Clinical Parasitology*, Philadelphia, 1937, Lea & Febiger.
2. Chandler, A. C.: *Introduction to Human Parasitology*, ed. 5, New York, 1936, John Wiley & Sons, Inc.
3. Hegner, R., Root, F. M., and Augustine, D. L.: *Animal Parasitology*, New York and London, 1929, Appleton Century Co.

---

## MASSIVE DOSES OF VITAMIN D IN CHRONIC ARTHRITIS: ITS EFFECT ON CALCIUM METABOLISM\*

CHARLES LEROY STEINBERG, M.D., ROCHESTER, N. Y.

THE first paper on the use of vitamin D in massive doses appeared in September, 1935.<sup>1</sup> In that publication Dreyer and Reed reported the results of two years' experience in the use of massive doses of vitamin D in 10 cases of infectious arthritis, 24 cases of atrophic arthritis, 14 cases of hypertrophic arthritis, 4 cases of menopausal arthritis, 3 cases of mixed arthritis, 5 cases of miscellaneous joint cases, and 7 cases of so-called arthralgia. Of this entire group 44 patients manifested clinical improvement, 13 patients were clinically unimproved, and in 10 patients the results were uncertain. These clinical investigators employed 200,000 to 1,000,000 units daily of vitamin D. The usual dose was 200,000 to 300,000 units daily.

---

\*From the Department of Medicine, Rochester General Hospital.  
Received for publication, January 21, 1938.

The next report, inspired by the first publication noted above, appeared in April, 1936.<sup>2</sup> Vrtiak and Lang reported the result of therapy with massive doses of vitamin D in 20 cases of atrophic arthritis in their study. They employed 150,000 to 300,000 units of vitamin D daily. The duration of treatment varied from three to ten months. Twelve of the patients showed a varied degree of clinical improvement. Eight of the 20 cases showed no improvement. The third report concerning this method of therapy appeared in October, 1936.<sup>3</sup> Wyatt and his co-workers reported on the use of 200,000 to 300,000 daily units of vitamin D in the treatment of 40 patients having atrophic arthritis. Eight of this group showed definite clinical improvement. The treatment failed in 24 cases. It was necessary to abandon the treatment in 8 cases because of unfavorable reactions. Thus clinical improvement has been reported in 46.7 per cent of 137 cases of arthritis, of which the majority was the atrophic type. More recently, Livingston<sup>4</sup> has reported good results by combining fever therapy with massive doses of vitamin D.

None of these investigators has reported any detailed study of the effects of massive doses of vitamin D on the serum calcium or serum phosphorus. They have been concerned chiefly with the clinical results. The effect of such therapy on the serum calcium and phosphorus was undertaken in this investigation.

Though much remains to be learned about normal calcium and phosphorus and even more is unknown of abnormal calcium-phosphorus metabolism, from the maze of calcium-phosphorus studies, several known facts have become established. In a normal subject the blood calcium is stabilized at 10 mg. per 100 c.c., the variation being 9 to 11.5. The serum phosphorus is stabilized in the neighborhood of 3 to 4 mg. per 100 c.c. The various factors which maintain these normal values and which upset them in a diseased state are not entirely known. What was thought to be one substance as regards a normal serum calcium is now thought to be three or four physiochemical divisions of this element. The previously accepted concept of these three states was that the serum calcium was made up of (1) a nondiffusible, nonionized fraction which was in loose combination with a serum protein (about 5 mg. per 100 c.c.); (2) a diffusible, nonionized fraction (about 3 mg. per 100 c.c.); and (3) a diffusible ionized fraction (about 2 mg. per 100 c.c.). The more recent concept divides the serum calcium into four fractions: two diffusible and two nondiffusible portions. McLellan and Hastings<sup>5</sup> are of the opinion that two of these fractions, the free ionized calcium and the calcium bound to the serum protein, are important to human economy.

Four or five factors operate to affect the serum calcium and phosphorus in a normal subject; these are (1) the calcium intake, (2) the phosphorus intake, (3) the hormone from the parathyroid gland, (4) vitamin D, and (5) the ferment phosphatase. There are some writers<sup>6</sup> who believe that the ingestion of calcium per se has no effect on the blood calcium; others believe contrawise. The latter group<sup>7</sup> claim that the period in which the blood is drawn for analysis after the calcium ingestion is most important. The phosphorus intake has a pronounced effect on the blood calcium. The ingestion of phosphorus in large doses in the form of sodium acid phosphate, has the following sequelae: (1) secretion of the excess phosphate in the urine, (2) a fall in an elevated serum

calcium, (3) an increase in output of fecal calcium, and (4) a fall in the calcium output in the urine. These sequelae result from the formation of insoluble calcium salts in the intestine.

The parathyroid hormone<sup>8</sup> raises the serum calcium and lowers the serum phosphorus, it increases the excretion of calcium and phosphorus in the urine, it increases the ionized calcium in the blood. The increased demand for calcium is either withdrawn from bony tissue or else supplied from a large amount of ingested calcium. Cantarow<sup>9</sup> has shown that vitamin D in excessive amounts causes a rise in the level of blood calcium or phosphorus or both. The extra calcium may come from increased absorption from the intestine, from better calcium utilization, or by withdrawal from the osseous tissue. The exact manner in which vitamin D brings about these changes is unknown. Some<sup>10</sup> believe that vitamin D stimulates the parathyroid tissue directly. However, there are many differences in the pharmacodynamics of vitamin D and parathyroid hormone which make the above hypothesis unlikely. The reader should refer to more detailed studies of calcium metabolism on this controversial subject.

Vitamin D also affects the ferment phosphatase, which is an ubiquitous substance in human economy. It has been found in such diverse tissues as the intestinal mucosa, kidney, bone, spleen, liver, pancreas, lung, cerebrum, muscle, milk, urine, bile, and others. It is chemically capable of splitting or hydrolyzing both aliphatic and aromatic esters of monophosphoric acid. Vitamin D has the power of restoring this ferment to the bone matrix where it eventually brings about precipitation of tricalcium phosphate for bone formation.

Our study concerns itself with the clinical effects observed in the use of massive doses of vitamin D in the treatment of chronic arthritis and the possible relationship of this effect to the changes in the blood calcium. We have administered this substance in usual doses of 160,000 USP units daily to 40 cases of chronic arthritis. Of the 29 cases of atrophic arthritis treated, 10 showed clinical improvement and 19 showed no improvement whatsoever. Of the 7 cases of hypertrophic arthritis treated, 2 showed clinical improvement, the results were questionable in one case, and failed in 4 cases. One case of Still's disease treated showed very definite improvement. Two cases of joint disease of unknown type, one of which showed marked osteoporosis around the shoulder girdle, and one case of arthralgia were definitely improved. One case of Marie Strumpell arthritis showed no clinical improvement by this method of treatment.

Thus, clinical improvement was noted in 14 cases or 35 per cent of the total treated, 26 cases or 65 per cent were unimproved. Three cases of the latter group were definitely aggravated. Of the 29 cases of atrophic arthritis improvement was noted in 10 cases or 34 per cent.

Initial serum calcium and phosphorus determinations were made in 32 cases. The average serum calcium in 25 cases of atrophic arthritis was 11.50 mg per 100 cc. Seventeen of this group had calcium values above 11 mg. Eight of this group had calcium values of 12 mg and more. The phosphorus determination in 23 cases of atrophic arthritis was normal with the exception of two instances, in which the readings were 2.7 and 2.9 mg, respectively. The average age of the male atrophic arthritic studied was thirty nine years, the range being

TABLE I

TABULAR STUDY OF DETAILED CALCIUM AND PHOSPHORUS DETERMINATIONS IN TWELVE CASES

CASE	HEMATOLOGY	CULTURES	INITIAL CALCIUM AND PHOSPHORUS	EFFECT MASSIVE DOSES VITAMIN D
62-year-old white female with marked osteoporosis of shoulder girdle. Pain in shoulder 18 months.	R.B.C. 3,980,000 Hb. 76 per cent Sahli W.B.C. 6,000 Sed. rate 0.62 Stabnuclears 6 per cent	Throat 60 per cent <i>Strep. hemolyticus</i>	Ca 11.7	At the end of 3 weeks treatment with daily doses of 160,000 units vit. D, Ca 13.5, P 4.9. No improvement. After 6 weeks such medication no improvement
43-year-old white male with abduction of arms limited to 20 degrees. Painful wrists and shoulders for 18 months.	R.B.C. 4,860,000 Hb. 94 per cent Sahli Sed. rate 1.55 Stabnuclears 3 per cent	Throat negative for <i>Strep. hemolyticus</i> . Skin tests positive for <i>Strep. viridans</i>	Ca 10.6 P 4.7	At the end of 2 weeks treatment with daily doses of 160,000 units vit. D, Ca 13.1, P 4.3. Patient able to use both shoulders in all ranges of action
33-year-old white female with typical atrophic arthritis of major joints of body for 8 months. No clinical results with vaccine therapy	Sed. rate 0.52 Stabnuclears 8 per cent R.B.C. 4,270,000 Hb. 86 per cent Sahli W.B.C. 8,000	Dental abscess <i>Strep. viridans</i>	Ca 10.7 P 4.2	No improvement at the end of 3 weeks treatment with daily doses of 160,000 units vit. D; 2 months later Ca 13.1. Definite clinical improvement
65-year-old white male with multiple joint pains for 6 years. Undiagnosed type of arthritis	R.B.C. 4,750,000 Hb. 94 per cent Sahli W.B.C. 8,000 Sed. rate 0.34 Stabnuclears 6 per cent	Throat negative for <i>Strep. hemolyticus</i>	Ca 9.7 P 4.8	At the end of 3 weeks treatment with daily doses of 160,000 units vit. D, Ca 10.8, P 2.8. Clinical improvement marked. 4 months later Ca 12, P 3.4. Improvement maintained
33-year-old white female with typical atrophic arthritis of several years' duration	Sed. rate 0.40 Stabnuclears 9 per cent R.B.C. 3,360,000 Hb. 67 per cent Sahli	Throat negative for <i>Strep. hemolyticus</i>	Ca 10.3 P 4.7	At the end of 3 weeks treatment with daily doses of 160,000 units vit. D, Ca 13.3, P 4.1; 1 month later Ca 13, P 3.9. Clinical improvement marked. 1 month later Ca 9.3, P 3.6; 1 month later Ca 11.8, P 3.2. Clinical improvement marked. 2 months later developed acute exacerbation and for the first time had flexion deformity of right elbow

32 to 49 years. The average age of the female atrophic arthritic was 45.3 years, the range being 32 to 79 years. The average blood calcium in 7 cases of hypertrophic arthritis was 11.2 mg. per 100 c.c. Two had values of 12 mg. or more and 5 of the group had a value about 11 mg. The average blood phosphorus in 6 cases of hypertrophic arthritis was 4.20 mg.

The finding of high normals of serum calcium and even hypercalcemia values in chronic arthritis, particularly in the atrophic type, in this series of cases

TABLE I—CONT'D

CASE	HEMATOLOGY	CULTURES	INITIAL CALCIUM AND PHOSPHORUS	EFFECT MASSIVE DOSES VITAMIN D
73 year old white female with flexion deformity of fingers and marked osteoporosis of hands	RBC 1,120,000 Hb 88 per cent Sdih Uric acid 2.7 Sed rate 105 Stabnuclears 11 per cent	Negative throat for <i>Strep hemolyticus</i>	Ca 17.2 P 3.5	At the end of 1 week treatment with daily doses of 160,000 units vit D, Ca 11.2, P 2.7. Improvement marked. At the end of 1 month Ca 17.6, P 3.5. At the end of 2 months Ca 13.2, P 4.2. 1 month later Ca 12.4, P 4.1. Clinical improvement maintained. Another 2 months and patient had regained complete motion of hands. Vit D stopped 3 months later. Ca 11.5, P 3.9. Patient feeling fine. Another 4 months later Ca 12.4, P 3.9. Vit D restarted 2 months later Ca 11.7, P 4.1. Clinical improvement maintained. No change in bone density.
35 year old white female with typical atrophic arthritis of several years' duration	RBC 3,960,000 Hb 79 per cent Sdih Sed rate 0.98 Stabnuclears 9 per cent	Tonsils negative for <i>Strep hemolyticus</i>	Not done	At the end of 3 weeks treatment with daily doses of 160,000 units vit D, Ca 12.2, P 3.3. 2 months later Ca 12, P 4.1. No clinical improvement. Another 2 months of treatment and joints definitely worse.
32 year old white female with typical atrophic arthritis for years	RBC 4,000,000 Hb 70 per cent Sdih Sed rate 0.80	Throat 60 per cent <i>Strep viridans</i>	Ca 10.9 P 3.9	At the end of 1 month treatment with daily doses of 160,000 units vit D, Ca 12.3, P 4.1. Joint condition definitely worse.
59 year old white female with advanced hypertrophic arthritis of spine	RBC 4,420,000 Hb 87 per cent Sdih WBC 16,200 Sed rate 0.49 Stabnuclears 10 per cent	Throat 75 per cent <i>Strep viridans</i>	Ca 8 P 3.6	At the end of 3 weeks treatment with daily doses of 160,000 units vit D, Ca 10.4, P 4. No improvement. At the end of another month treatment Ca 12, P 4.1. No improvement.

needs further explanation. It is true that no agreement exists as to whether or not a normal or abnormal blood calcium is characteristic of chronic arthritis. Some investigators<sup>13</sup> have reported frequent elevations of the blood calcium in various forms of chronic arthritis, others<sup>14</sup> have found normal calcium values in 97 per cent of chronic arthritides studied. The explanation of these differences in opinion probably can be explained on the vicissitudes of the pathologic process

TABLE I

TABULAR STUDY OF DETAILED CALCIUM AND PHOSPHORUS DETERMINATIONS IN TWELVE CASES

CASE	HEMATOLOGY	CULTURES	INITIAL CALCIUM AND PHOSPHORUS	EFFECT MASSIVE DOSES VITAMIN D
62-year-old white female with marked osteoporosis of shoulder girdle. Pain in shoulder 18 months.	R.B.C. 3,980,000 Hb. 76 per cent Sahli W.B.C. 6,000 Sed. rate 0.62 Stabnuclears 6 per cent	Throat 60 per cent <i>Strep. hemolyticus</i>	Ca 11.7	At the end of 3 weeks treatment with daily doses of 160,000 units vit. D, Ca 13.5, P 4.9. No improvement. After 6 weeks such medication no improvement
43-year-old white male with abduction of arms limited to 20 degrees. Painful wrists and shoulders for 18 months.	R.B.C. 4,860,000 Hb. 94 per cent Sahli Sed. rate 1.55 Stabnuclears 3 per cent	Throat negative for <i>Strep. hemolyticus</i> . Skin tests positive for <i>Strep. viridans</i>	Ca 10.6 P 4.7	At the end of 2 weeks treatment with daily doses of 160,000 units vit. D, Ca 13.1, P 4.3. Patient able to use both shoulders in all ranges of action
33-year-old white female with typical atrophic arthritis of major joints of body for 8 months. No clinical results with vaccine therapy	Sed. rate 0.52 Stabnuclears 8 per cent R.B.C. 4,270,000 Hb. 86 per cent Sahli W.B.C. 8,000	Dental abscess <i>Strep. viridans</i>	Ca 10.7 P 4.2	No improvement at the end of 3 weeks treatment with daily doses of 160,000 units vit. D; 2 months later Ca 13.1. Definite clinical improvement
65-year-old white male with multiple joint pains for 6 years. Un-diagnosed type of arthritis	R.B.C. 4,750,000 Hb. 94 per cent Sahli W.B.C. 8,000 Sed. rate 0.34 Stabnuclears 6 per cent	Throat negative for <i>Strep. hemolyticus</i>	Ca 9.7 P 4.8	At the end of 3 weeks treatment with daily doses of 160,000 units vit. D, Ca 10.8, P 2.8. Clinical improvement marked. 4 months later Ca 12, P 3.4. Improvement maintained
33-year-old white female with typical atrophic arthritis of several years' duration	Sed. rate 0.40 Stabnuclears 9 per cent R.B.C. 3,360,000 Hb. 67 per cent Sahli	Throat negative for <i>Strep. hemolyticus</i>	Ca 10.3 P 4.7	At the end of 3 weeks treatment with daily doses of 160,000 units vit. D, Ca 13.3, P 4.1; 1 month later Ca 13, P 3.9. Clinical improvement marked. 1 month later Ca 9.3, P 3.6; 1 month later Ca 11.8, P 3.2. Clinical improvement marked. 2 months later developed acute exacerbation and for the first time had flexion deformity of right elbow

32 to 49 years. The average age of the female atrophic arthritic was 45.3 years, the range being 32 to 79 years. The average blood calcium in 7 cases of hypertrophic arthritis was 11.2 mg. per 100 c.c. Two had values of 12 mg. or more and 5 of the group had a value about 11 mg. The average blood phosphorus in 6 cases of hypertrophic arthritis was 4.20 mg.

The finding of high normals of serum calcium and even hypercalcemia values in chronic arthritis, particularly in the atrophic type, in this series of cases

specificity of action which might be ascribed to this method of therapy. Sixty-eight calcium determinations and 64 phosphorus studies were made on this group. The diets in all these cases were similar. All received 1000 cc of milk daily, 4 cups of 5 per cent and 10 per cent vegetables, 240 gm of 10 per cent fruit, 60 gm of whole wheat bread, 1 gm protein per kg, and sufficient butter fat to make up the caloric requirement.

A study of Table I suggests that massive doses of vitamin D have the following sequelae on the blood calcium. It will lower a high serum calcium, it will raise a low or normal serum calcium to a higher level and then, after continued administration, will again decrease the hypercalcemia level. The clinical improvement or nonimprovement or even aggravation of existing joint symptoms had no bearing on the change of blood calcium, for in some cases with the production of a hypercalcemia, clinical improvement would be noted, and in others an opposite effect or no improvement would occur. The effect on the serum phosphorus was less marked.

It appears that the effect of massive doses of vitamin D in chronic arthritis is nonspecific, and that the improvement noted with this medication would fall in line with the many other remedies employed in the nonspecific treatment of chronic arthritis.

Although massive doses of vitamin D were administered in 40 cases of chronic arthritis in periods, ranging from several weeks to one and one half years, and were also given to a child aged 3 to 4 years in doses of 160,000 units daily, no untoward results occurred. Even though the author feels that no specific virtues exist in such medication, at the same time he feels that its toxicity has been overemphasized. The above statement is not meant to imply that larger doses than employed here may not be dangerous or even fatal. The diets in all our cases were controlled. If the calcium intake had been increased, or acid substances such as ammonium chloride given, hypervitaminosis most probably would have resulted. The clinical investigator should beware of the following symptoms in the use of massive doses of vitamin D: nausea, anorexia, vomiting, cramps, diarrhea, thirst, and frequent urination. Also peripheral nerve changes, pain in muscles and joints, headaches, haziness, and vertigo are all danger signals of hypervitaminosis D. The drug should be stopped as soon as any of these symptoms occur. It may be restarted ten to fourteen days later.

The concentrated vitamin D used in this study was supplied through the courtesy of the experimental medical department, Parke Davis & Co.

#### REFERENCES

1. Dreyer, I., and Reed, C. I. The Treatment of Arthritis With Massive Doses of Vitamin D, *Arch. Phys. Therapy* 16: 537, 1935.
2. Vrtiak, E. G., and Lang, R. S. Observations on the Treatment of Chronic Arthritis With Vitamin D, *J. A. M. A.* 106: 1162, 1936.
3. Wyatt, B. L., Hicks, R. A., and Thompson, H. E. Massive Doses of Vitamin D in the Treatment of Proliferative Arthritis, *Ann. Int. Med.* 10: 427, 1936.
4. Livingston, S. K. Vitamin D and Fever Therapy in Chronic Arthritis, *Arch. Phys. Therapy* 17: 704, 1936.
5. McLean, F. C., and Hastings, A. B. The State of Calcium in the Fluids of the Blood, *J. Biol. Chem.* 108: 285, 1935.



6. Denis, W., and Minot, A. S.: Effects of Feeding With Calcium Salts on the Calcium Content of the Blood, *J. Biol. Chem.* 41: 357, 1920.
7. Kohn, B. S., and Roe, J. H.: Calcium Absorption From the Intestinal Tract in Human Subjects, *J. A. M. A.* 86: 1761, 1926.
8. Aub, J. C.: Parathyroid Hormone Therapy, *J. A. M. A.* 105: 197, 1935.
9. Cantarow, A.: Review of Phosphatase Activity, *Internat. Clin.* 1: 246, 1936.
10. Taylor, N. B., Weld, C. B., and Sykes, J. F.: Relation of Parathyroid Glands to Action of Irradiated Ergosterol, *Proc. Roy. Soc., London, S. B.* 116: 10, 1934.
11. Jones, R. W., and Roberts, R. E.: Calcification, Decalcification, and Ossification, *Brit. J. Surg.* 21: 461, 1934.
12. Mark, R.: Arthritis Deformans and Calcium Metabolism, *J. Metab. Research* 4: 135, 1924.
13. Horowitz, P.: The Calcium Content of the Blood in Gout and Arthritis, *Am. J. M. Sc.* 171: 560, 1926.
14. Hartung, E. F., and Greene, C. H.: Serum Calcium in Arthritis, *J. LAB. & CLIN. MED.* 20: 929, 1935.

## STUDIES ON THE ANEMIA OF PREGNANCY IN GASTRECTOMIZED AND NORMAL DOGS\*

R. A. BUSSABARGER, M.D., PH.D.,† F. P. CUTHBERT, M.S., M.D., AND  
A. C. IVY, PH.D., M.D., CHICAGO, ILL.

THE anemias of pregnancy have been recently reviewed by several authors.<sup>1-7</sup> They reported that "secondary" anemia, a hypochromic microcytic anemia, was the most common of the true anemias of pregnancy. This anemia was found to occur in from 30 to 40 per cent of all pregnant women.

Ivy, Morgan and Farrell<sup>8</sup> in 1931 reported the occurrence of a spontaneous anemia in completely gastrectomized dogs. They also reported a few instances of anemia of pregnancy in gastrectomized dogs. The spontaneous anemia and the anemia of pregnancy were "secondary" anemias. These authors concluded that in the gastrectomized dog the factor of safety in digestion was so impaired that the added strain of pregnancy caused an anemia. To further investigate this anemia of pregnancy in the gastrectomized dog, the following studies were made.

Five gastrectomized dogs were observed through a total of fifteen pregnancies. A pregnancy which occurred after a five-foot enterectomy in one gastrectomized dog and a pregnancy which occurred after a six-foot enterectomy in another gastrectomized dog were studied. As a control series, twelve normal dogs were observed through twelve pregnancies.

### METHODS

The five gastrectomized dogs used in this study were under observation in this laboratory for periods varying from two to seven years. When this study was first undertaken, hematologic procedures were confined to determination of red blood count and hemoglobin; but these studies were later extended to include determinations of hematocrit, blood volume, and, in two cases, mean cell diameters. All animals, including the gastrectomized dogs, received the

\*From the Department of Physiology and Pharmacology, Northwestern University Medical School.

†Jessie Horton Koesler Fellow in Physiology of the Institute of Medicine, Chicago.

Received for publication, January 27, 1938.

same stock laboratory diet made up of boiled hamburger, white bread, prepared cereal, milk, and cod liver oil

*Hematologic Procedures*—With the exception of the first seven pregnancies observed in the gastrectomized dogs, blood examinations consisting of red blood count, hemoglobin by the Newcomber method, hematocrit by Rosalin's method,<sup>9</sup> and blood volume by the method of Hopper and associates<sup>10</sup> were made at intervals of approximately ten days. Examinations were made more frequently just prior to and just following delivery.

### RESULTS

Standard values for red blood count, hemoglobin and hematocrit established for normal dogs in our laboratory compare favorably with those accumulated by Wintrobe and co-workers.<sup>11</sup> For purposes of conciseness and clarity, arbitrary limits for slight, moderate, marked, and severe anemia were established. The definitions of these limits follow.

ANEMIA	RB C	Hb	HEMATOCRIT	FRACTION OF NORMAL
Normal	7.00	15.00	41.50	6/6
Slight	5.83	12.50	38.70	5/6
Moderate	4.67	10.00	1.00	4/6
Marked	3.50	7.00	23.20	3/6
Severe	2.33	5.00	15.50	2/6

*Gastrectomized Dogs*—Gastrectomized Dog No. 11 was observed during eight pregnancies. In five pregnancies (No. 11 1, 2, 3, 6, 7) hypochromic anemia gradually developed in the last thirty days of pregnancy to become marked during the week preceding delivery. In one pregnancy, No. 11 4, a marked normochromic anemia and in another pregnancy, No. 11 5, a severe hypochromic anemia were present at term. The eighth and last pregnancy observed in this dog occurred after a five foot enterectomy. This pregnancy was terminated by a fatal hemorrhage caused by uterine rupture from pressure necrosis.

In pregnancy No. 11 3 daily feeding of one vial of liver extract No. 343 (10 cc from 50 gm liver) Lilly instituted on the sixteenth ante partum day resulted in a marked increase in red blood count, but had no effect upon the hemoglobin level of the blood. In pregnancy No. 11 4 daily feeding of 10 gm of liver extract No. 55 Lilly instituted upon the sixteenth ante partum day had no demonstrable effect on the blood picture.

In pregnancy No. 11 5 the blood volume at term was increased by approximately 240 cc and the total circulating hemoglobin decreased by approximately 50 gm, in pregnancy No. 11 6 the blood volume was increased by approximately 350 cc and the total circulating hemoglobin decreased by approximately 25 gm, and in pregnancy No. 11 7 the blood volume was increased by approximately 100 cc and the total circulating hemoglobin decreased by approximately 25 gm.

Gastrectomized Dog No. 26 was observed during three pregnancies. In pregnancy No. 26 1 a marked hypochromic anemia, in pregnancy No. 26 2 a

## CONDENSED PROTOCOLS

*Gastrectomized Dog No. 11*

NO. OF PREG. AND TIME AFTER OPERA- TION	DAYS ANTE OR POST PARTUM	WT. LBS.	R.B.C. MIL- LIONS	HB. GM.	HE- MATO- CRIT	COLOR INDEX	BLOOD VOLUME	TOTAL HB.	MIS- CELLANEOUS
1st 3 mo. p.o.	55 a.p. 0 15 p.p. 64 p.p.		5,70 3,44 3,90 5,50	13.10  8.15 11.80		1.07  0.97 1.00			Premature de- livery; 6 or 7 wk. preg- nant
2nd 1 yr. p.o.	49 a.p. 20 a.p. 0 15 p.p. 79 p.p.		6,00 4,90 4,70 5,50 6,70	12.80 8.70 7.80 9.70 14.40		1.00 0.83 0.78 0.82 1.00			Premature de- livery; dead fetuses
3rd 1 yr. 6 mo. p.o.	46 a.p. 16 a.p. 1 a.p. 15 p.p. 81 p.p.		6,80 4,20 6,90 5,40 4,54	11.60 8.00 7.78 9.50 8.80		0.80 0.89 0.53 0.82 0.90			Term; 11 pups  1 vial liv. ext. No. 343 daily
4th 2 yr. 1 mo. p.o.	45 a.p. 16 a.p. 0 21 p.p. 42 p.p.		5,70 4,10 4,20 4,60 6,73	12.05 10.20 8.70 10.10 13.10	40.50 26.20 22.80 30.40 34.60	0.99 1.16 0.97 1.03 0.91			Term; 10 pups  1 vial liv. ext. No. 55 daily
5th 2 yr. 7½ mo. p.o.	55 a.p. 10 a.p. 1 a.p. 18 p.p. 37 p.p.	40½ 43½ 49¾ 42¼ 46	6,05 4,49 4,30 5,29 6,91	12.56 8.54 6.97 11.10 12.90	29.90 27.50 26.00 28.90 41.10	0.97 0.89 0.75 0.98 0.87	1178 1314 1420 1223 1255	148 112 99 136 162	Premature de- livery; dead fetuses
6th 3 yr. 1 mo. p.o.	59 a.p. 15 a.p. 0 13 p.p. 72 p.p.	46¾ 48 47¾	6,98 5,69 4,83 4,69 6,52	12.63 10.03 8.42 8.21 11.38	34.90 25.90 25.90 25.90 36.50	0.84 0.82 0.81 0.82 0.82	1194 1450 1450 1230	151 122 140	Premature de- livery; dead fetuses
7th 4 yr. 2½ mo. p.o.	59 a.p. 16 a.p. 0 1 p.p. 17 p.p. 47 p.p.	47	5,57 5,02 4,55 4,58 6,03 7,85	12.77 11.19 8.90 9.90 9.87 11.19		1.07 1.10 0.91 0.91 0.77 0.67			Term; 4 pups

Seven years after gastrectomy No. 11 was subjected to a 5 ft. enterectomy.

Compo-site of 4 determi- nations before opera- tion		44	5,64	11.21	37.75	0.93			
8th 181 days after enterectomy	59 a.p. 26 a.p. 8 a.p. 1 a.p. Dead	35 32¾ 34½ 32	4,60 4,03 4,26 4,45	11.48 9.85 8.63 9.71	37.00 34.00 28.00 31.00	1.16 1.14 0.94 1.02	1160 1090 1090 993	133 107 94 96	

Pressure necrosis with rupture of the uterus  
Five fetuses approximately one week from term

moderate normochromic anemia, and in pregnancy No. 25-3 a moderate hypochromic anemia developed. In the last pregnancy, No. 26-3, the blood volume changed very slightly, but a marked decrease in total circulating hemoglobin occurred.

The one pregnancy observed in gastrectomized Dog No. 27 had to be terminated by Porro section, approximately one and one-half weeks from term. Approximately two weeks before term this animal developed a severe hyperchromic anemia and became too weak to stand. After a transfusion of

CONDENSED PROTOCOLS  
Gastrectomized Dog No 26

NO OF PREG AND TIME AFTER OPERA- TION	DAYS ANTE OF POST PARTUM	WT LBS	RBC MILLIONS	HB GM	HE MATO- CRIT	COLOR INDEX	BLOOD VOLUME	TOTAL HB	MIS- CELLANEOUS
1st 2 mo po	58 ap 7 ap 17 ap 0 22 pp 55 pp 71 pp		6.80 5.80 6.00 5.00 6.50 7.15 7.85	14.00 12.70 11.70 8.65 10.30 12.50 14.20		0.96 1.02 0.91 0.81 0.74 0.82 0.84			Term, 12 pups
2nd 8 mo po	54 ap 19 ap 3 ap 18 pp 91 pp		6.50 6.40 5.80 6.40 7.23	12.80 12.20 11.70 12.05 15.08	45.00 38.80 39.00 39.60 50.20	0.92 0.89 0.94 0.88 0.97			Term, 3 pups
3rd 14 mo po	55 ap 29 ap 15 ap 1 ap 11 pp 48 pp 98 pp	46½ 46½ 42½ 45 47½	7.23 7.99 6.49 6.60 5.87 6.72 7.90	15.05 14.03 11.82 11.59 12.17 14.73 15.08	47.40 44.70 42.70 40.70 40.70 44.70 47.30	0.97 0.82 0.85 0.82 0.97 1.02 0.89	1504 1555 1405 1697	211 184 171 256	Near term, 7 pups born dead

Gastrectomized Dog No 27

1st 1 mo po	65 ap 11 ap 17 ap 0 1 pp 30 pp 48 pp 50 pp		7.50 8.00 6.80 2.95 4.90 4.57 3.30 Dead	11.30 11.90 11.30 8.30 10.70 9.05 8.00 Diffuse peritonitis		0.70 0.70 0.78 1.31 1.02 0.92 1.13			Transfused with 200 cc whole blood Porro section —9 pups 1½ weeks from term
----------------	---	--	--	---	--	--	--	--	---

Gastrectomized Dog No 33

1st 5½ mo po	57 ap 24 ap 0 20 pp 44 pp	29½ 33½ 35½ 30	7.65 6.30 4.25 7.67 7.06	12.44 11.19 6.88 9.36 12.40	36.2 35.0 27.0 26.5 35.5	0.76 0.83 0.75 0.77 0.76	848 817 892 908	106 91 61 85	6.27 µ 5.96 µ 4.36 µ 5.94 µ	Term 10 pups
-----------------	---------------------------------------	-------------------------	--------------------------------------	---	--------------------------------------	--------------------------------------	--------------------------	-----------------------	--------------------------------------	--------------------

2½ years after gastrectomy No 33 was subjected to a b ft enterectomy

Composite of 4 determi- nations before opera- tion		28	6.80	12.93	46.44	0.89			
2nd 83 days after enterectomy	91 ap 43 ap 9 ap 4 pp 43 pp 154 pp	26½ 25½ 27½ 23½ 26 25½	7.15 6.45 4.67 5.15 5.82 7.99	14.72 13.14 10.50 10.30 12.52 16.32	42.50 46.00 37.50 33.25 41.50 53.50	0.96 0.95 1.05 0.93 1.00 0.96	1055 1050 1020 925 1050 930	155 138 107 95 132 152	Term, 3 pups

300 cc of blood, the operation was successfully performed, but the animal died fifty days later. Autopsy revealed multiple abscesses of the liver and lungs and a diffuse peritonitis.

Gastrectomized Dog No 31 was observed during three pregnancies. In pregnancy No 31 1 a slight hypochromic anemia developed. The blood volume at term was decreased by approximately 300 cc and the total circulating hemoglobin by approximately 90 gm. In pregnancy No 31 2 a marked hypochromic anemia developed. The blood volume at term was decreased by approximately 400 cc and the total circulating hemoglobin by approximately

CONDENSED PROTOCOLS  
Gastrectomized Dog No. 31

NO. OF PREG. AND TIME AFTER OPERA- TION	DAYS ANTE OR POST PARTUM	WT. LBS.	R.B.C. MIL- LIONS	HB. GM.	HE- MATO- CRIT	COLOR INDEX	BLOOD VOLUME	TOTAL HB.	MIS- CELLANEOUS
Before gastrectomy		39½	7,60	15.43	56.00	0.95	1540	238	
1st	70 a.p.	42½	8,16	15.10	60.40	0.86			Term; 8 pups
4 mo. 1 wk.	15 a.p.	49	6,95	14.38	39.80	0.97	1296	186	
p.o.	2 a.p.	55	6,33	12.51	35.50	0.92	1240	155	
	17 p.p.		6,07	11.10		0.85			
	79 p.p.	48	9,13	15.78	45.50	0.81	1553	245	
2nd	65 a.p.	Same as 79 p.p. of first pregnancy							
8¾ mo. p.o.	47 a.p.		9,16	16.91		0.86			Term; 7 pups
	13 a.p.		8,28	13.50		0.76			
	0		5,69	7.52		0.62			
	3 p.p.	41½	5,83	8.15	28.60	0.65	1150	94	
	23 p.p.		7,20	10.36		0.67			
	82 p.p.	39½	8,74	12.17	39.60	0.65	1077	131	
3rd	62 a.p.	43	8,11	12.57	32.00	0.72	1125	141	5.94 μ
1¾ yr. p.o.	17 a.p.		8,69	11.23	29.50	0.60			6.04 μ Term; 9 pups
	6 a.p.	49½	8,19	8.49	27.00	0.48	1470	124	
	1 p.p.		7,36	8.28	24.00	0.53			
	32 p.p.		9,67	10.20	38.00	0.49			

155 gm. In pregnancy No. 31-3 a marked hypochromic anemia developed. The blood volume at term was increased by approximately 350 c.c., and the total circulating hemoglobin decreased by approximately 20 gm. The mean cell diameter preceding this pregnancy was 6.21  $\mu$ . The mean cell diameter at term was 6.04  $\mu$ .

Gastrectomized Dog No. 33 was observed during one pregnancy. A severe hypochromic anemia developed. The blood volume at term remained unchanged, but the total circulating hemoglobin was decreased by approximately 44 gm. The mean cell diameter prior to pregnancy was 6.27  $\mu$ , at mid-term 5.96  $\mu$ , and at term 4.36  $\mu$ . Gastrectomized Dog No. 33 was subjected to an enterectomy of six feet of jejunum and ileum. Two weeks after the operation the animal had a moderate hypochromic anemia; but from the thirtieth post-operative day to the one hundred and fourteenth postoperative day, when the animal again became pregnant, the blood picture showed no anemia. During this pregnancy the animal developed a moderate normochromic anemia, the blood volume remained unchanged; but the total circulating hemoglobin decreased by approximately 45 gm.

*Normal Dogs.*—Each of 12 normal dogs were observed during one pregnancy. The blood picture at term showed a slight normochromic anemia in 5 animals (Nos. 1, 3, 5, 6, and 9), slight hypochromic anemia in one animal (No. 10), moderate normochromic anemia in 3 animals (Nos. 0, 2, and 7), moderate hypochromic anemia in 2 animals (Nos. 8 and 11), and marked normochromic anemia in one animal (No. 4).

In 8 cases in which blood volume determinations were performed a sufficient number of times to be adequate, the blood volume at term was unchanged in 4 cases, Nos. 3, 6, 7, and 10; increased in 3 cases by approximately 175 c.c. in Case No. 0, 300 c.c. in Case 2, and 100 c.c. in Case 5; and decreased in one case, No. 4, by approximately 130 c.c. Of these 8 cases the total circulating

## CONDENSED PROTOCOLS

## Normal Control Dogs

NO OF DOG	DAYS ANTE OP POST PARTUM	WT LBS	PBC MH LIONS	HB GM	HE MATO CPIT	COLOP INDEX	BLOOD VOL UME	TOTAL HB	MISCEL LANEOS
0	0	28	5.56	12.22	37.00	0.97	980	120	Term
	1 pp	23	4.70	10.15	28.50	1.01	755	77	9 pups
	25 pp	21½	4.85	11.61	39.25	1.12	840	97	
	29 pp	21	5.05	11.88	38.00	1.10	845	100	
	64 pp	22½	7.35	14.93	50.00	0.95	805	120	
1	1 ap	37	6.08	12.67	38.75	0.97	1023	130	Term
	2 pp	32	5.39	11.78	34.00	1.02	893	105	7 pups
	25 pp	24	5.00	10.94	35.00	1.02	822	90	
Very sick with snuffles Killed									
2	5 ap	46	4.40	10.20	20.00	1.08	1360	139	
	4 ap	46	4.44	10.20	30.50	1.07	1350	138	Term
	2 pp	38	4.75	10.25	20.50	1.01	1170	120	5 pups
	26 pp	32½	4.28	9.80	31.00	1.07	1190	117	
	52 pp	30½	4.85	11.88	35.00	1.14	1040	123	
3	55 ap	17½	6.99	14.40	38.80	0.96	408	63	
	19 ap		6.29	14.30		1.06			
	5 ap		6.50	14.60		1.05			
	0	18½	6.45	14.20	38.00	1.03	450	64	Term
	11 mo pp	18	6.99	17.47	29.20	0.90	472	64	5 pups
4	9 ap	1	4.28	9.45	28.00	1.03	1110	105	
	6 ap	24	4.00	8.89	25.50	1.04	1080	96	Term
	1 pp	26	3.56	8.25	25.00	1.08	904	75	8 pups
	92 pp	29½	7.07	12.75	45.00	0.83	1210	154	
5	0	28½	6.08	13.94	44.50	1.07	1190	166	Term
	1 pp	22½	4.82	11.00	32.50	1.07	865	95	9 pups
	29 pp	31	6.91	14.72	46.50	1.00			
	117 pp	26½	7.25	14.41	48.00	0.93	1090	157	
6	1 ap	47½	6.57	14.28	45.00	1.01	1570	195	Term
	4 pp	38½	5.21	11.48	36.00	1.03	1350	155	8 pups
	28 pp	31½	6.90	12.30	39.50	0.83	1300	160	
	80 pp	22	6.65	13.94	45.00	0.98	1360	190	
	96 pp	33	7.28	12.98	40.50	0.83	1430	186	
7	19 ap	25	5.75	13.49	37.00	1.09	777	105	
	2 ap	25	5.91	11.80	24.50	0.93	773	91	Term
	1 pp	21	4.60	10.20	29.25	1.03	825	84	7 pups
	6 pp	18½	4.21	9.16	28.00	1.01	727	67	
	52 pp	21	6.77	13.66	44.00	0.94	743	101	
	55 pp	20	6.59	13.06	40.00	0.92	777	102	
8	4 ap	49	5.36	12.32	35.75	1.09	1700	213	
	1 ap	50	6.40	11.75	37.50	0.86			Term
	1 pp	43	5.50	11.24	36.50	0.95	1760	198	5 pups
	24 pp	33	4.93	10.10	33.00	0.96	1450	147	
	42 pp								
Dead Lobar pneumonia									
9	6 ap	14	6.17	14.72	44.50	1.11			
	4 ap	14½	6.39	13.06	42.00	0.96	379	49	Term
	3 pp	10½	5.14	8.75	32.00	0.79			5 pups
10	62 ap	21½	7.28	14.73	46.00	0.95	763	113	Term
	41 ap	20	7.26	13.75	44.40	0.88	735	101	5 pups
	13 ap	23	6.10	10.90	48.20	0.83	784	86	
	5 ap		6.54	12.63		0.90			
	2 pp		6.01	11.71		0.91			
	18 pp		7.09	11.81		0.78			
11	46 ap	24½	7.43	10.62	42.50	0.73	800	85	Term
	22 ap	24½	6.72	11.59	43.10	0.80	802	93	6 pups
	8 ap		6.42	11.94		0.87			
	1 pp	25	6.35	9.43	41.60	0.69	820	77	
	41 pp		7.85	13.89		0.83			

hemoglobin at term showed very little change with one exception. In Case 4, which showed approximately 130 c.c. decrease in blood volume at term, the total circulating hemoglobin was decreased by approximately 58 gm.

*Summary of Results.*—With few exceptions, pregnancy in the gastrectomized dog resulted in a marked or severe anemia. Blood volume determinations made in eight pregnancies demonstrated in every case a total circulating hemoglobin decrease of from 20 to 155 gm. regardless of changes in blood volume. In two gastrectomized dogs subjected to extensive enterectomy, the anemia of pregnancy, as judged from the blood picture alone, appeared to be no more severe than before enterectomy. However, in one case the size of the litter was much less and in the other case dehydration supervened. In both cases the total circulating hemoglobin was markedly reduced.

In general, the severity of the anemia appeared to be related to the size of the litter and to the shortness of the interval between successive pregnancies.

Pregnancy in normal dogs caused a marked anemia in only one case. Hydremia occurred in only 3 out of 8 cases (38 per cent). Anemia of the puerperium was more marked than the anemia of pregnancy in almost every case.

#### DISCUSSION

The above results indicate that hydremia of pregnancy occurs in approximately one-third of normal dogs and that anemia of pregnancy in normal dogs is uncommon. These findings are in accord with those of earlier authors. Spiegelberg and Gescheidler<sup>12</sup> in 1872 and Nasse<sup>13</sup> in 1876 reported that in dogs the blood was more dilute during the last half of pregnancy. Spiegelberg and Gescheidler<sup>12</sup> did not believe this significant because they found that the percentage of hemoglobin in pregnant and nonpregnant dogs was equal. In 1927, Schmidt and associates<sup>14</sup> reported that a slight hydremia occurred during the last ten days of pregnancy in the dog. They believed that the extent of the hydremia was significant, but they reported the results of only three experiments.

The occurrence of a marked to severe anemia which developed during the last half of pregnancy in the gastrectomized animals appears easily explicable in the light of several reports. Mullenix,<sup>15</sup> Dragstedt,<sup>16</sup> Kellogg,<sup>17</sup> and Mettier<sup>18</sup> and their co-workers have observed that the production capacity for hemoglobin regeneration is markedly reduced in the gastrectomized dog. These authors also found the production capacity for hemoglobin regeneration was markedly increased by the administration of iron. Moreover, Hugouenq<sup>19</sup> reported that in the case of the human fetus two-thirds of the storage of iron takes place during the last three months before birth. Nicholas<sup>20</sup> observed that the storage of hemoglobin in rat fetuses is most rapid during the early part of the last third of the gestation period. And Zeidberg<sup>21</sup> found that in the rabbit the last half of pregnancy is the period during which the fetus is laying down its chief supply of blood-building materials. These facts, along with the fact that anemia was most marked in those gastrectomized dogs bearing the largest litters, lead us to believe that in the presence of a markedly reduced production capacity for hemoglobin regenera-

tion the added strain of fetal demand for blood building materials uniformly brings about a marked to severe anemia of pregnancy in the gastrectomized dog.

In the light of these experiences let us examine the various hypotheses advanced to explain anemia of pregnancy in women. Strauss and Castle<sup>2</sup> studied gastric secretion during pregnancy and puerperium in 24 women and found that 75 per cent of these women did not secrete normal amounts of free hydrochloric acid and pepsin during more than half of the period of pregnancy. From this and other studies these authors concluded that the hypochromic anemia of pregnancy is due either to a direct dietary deficiency or to a deficiency conditioned by gastric anaacidity, hypoacidity, or associated gastrointestinal defects in the presence of fetal demand for blood-building materials. The importance of a gastric secretory defect in the development of hypochromic anemia of pregnancy has been supported by the observations of Rowland,<sup>1</sup> Davies and Shelley,<sup>22</sup> and Goodall and Gottlieb.<sup>23</sup> At first glance, the development of a marked to severe anemia of pregnancy in gastrectomized dogs, but not in normal dogs seems to lend experimental support to the hypothesis that gastric defects play an important role in the development of a hypochromic anemia of pregnancy. However, Lyon<sup>24</sup> and Fullerton<sup>25</sup> believe that marked hypochromic anemia of pregnancy is probably dependent upon the presence of a pre-existing anemia which was not necessarily dependent upon pregnancy per se. Bethell<sup>26</sup> has gone further to point out that the pregnant woman saves, from the absence of menstrual blood loss over ten periods, from one third to one half of the amount of iron utilized by the fetus. He states that the reserves of blood and blood building materials possessed by the healthy person without a tendency toward anemia are adequate to replace the net iron loss from mother to fetus without demonstrable effect upon the blood level. Bethell concluded from his studies that anemia in pregnancy is commonly due either to pre-existing iron depletion or to an inadequate intake of protein of high biologic value during gestation.

Do these factors play a part in the anemia of pregnancy of the gastrectomized dog? Mettler and Minot<sup>27</sup> have reported that iron is more readily absorbed from an acid than from an alkaline medium. Caryl and co-workers<sup>28</sup> and Emery<sup>29</sup> have shown that digestion and assimilation of protein are markedly impaired in the gastrectomized dog. Moreover, as has been mentioned before, Ivy, Morgan, and Farrell<sup>8</sup> reported the occurrence of spontaneous anemia in the gastrectomized dog. Therefore, the reserve stores of blood building materials in the gastrectomized dog are, presumably, low. Since normochromic anemia of pregnancy occurred in some gastrectomized dogs (i.e., No 113 and No 262) and in two gastrectomized and partially enterectomized dogs (No 118 and No 332), the factors predisposing to anemia emphasized by Bethell<sup>26</sup> seem especially significant in explaining the anemia of pregnancy in the gastrectomized dog.

Since the achylia gastrica produced by gastrectomy is permanent and leads to a markedly decreased reserve of blood building materials and to a tendency to spontaneous anemia, the results of the experiments reported here may or may not lend support to the hypothesis that transient gastric secretory defects in pregnant women may cause an anemia of pregnancy. Likewise, from these



experiments, little can be said about the role of the stomach in causing "pernicious anemia" of pregnancy because Richter and associates<sup>30</sup> and Ivy and others<sup>31</sup> have shown that canine stomach is inactive in pernicious anemia. In addition, Bussabarger, Ivy, and Richter<sup>32</sup> have found the entire intestine of the dog to be inactive in pernicious anemia. In view of the fact that the hog duodenum<sup>33, 34</sup> and hog colon<sup>35</sup> have been shown to be active in pernicious anemia, it would seem that too much attention has been focused upon the stomach at the expense of the rest of the gastrointestinal tract.

#### SUMMARY AND CONCLUSIONS

1. The blood picture of five gastrectomized dogs was observed through a total of fifteen pregnancies. (a) Marked to severe anemia developed in the gastrectomized dogs in eleven pregnancies. (b) Blood volume determinations made in eight pregnancies demonstrated in every case a decrease in total circulating hemoglobin of from 20 to 155 gm., regardless of changes in the blood volume. (c) In two gastrectomized dogs subjected to extensive enterectomy the anemia of pregnancy, as judged by the blood picture alone, appeared to be less severe than before the enterectomy. However, in both cases the total circulating hemoglobin was markedly reduced.

2. The blood picture of twelve normal dogs was observed through twelve pregnancies. (a) Marked anemia of pregnancy developed in only one case. (b) Anemia of the puerperium was more marked than anemia of pregnancy. (c) Blood volume determinations made in eight pregnancies disclosed hydreemia in only three cases.

3. The etiology of anemia of pregnancy was discussed.

#### REFERENCES

- Rowland, V. C.: The Pernicious or Hemolytic Anemia of Pregnancy, *J. A. M. A.* 82: 372, 1924.  
Anemia of Pregnancy. Relation to Anemia in General, *Ibid.* 100: 537, 1935.
- Strauss, M. B., and Castle, W. B.: Studies of Anemia in Pregnancy. I. Gastric Secretion in Pregnancy and the Puerperium, *Am. J. M. Sc.* 134: 655, 1932.  
II. The Relationship of Dietary Deficiency and Gastric Secretion to Blood Formation During Pregnancy, *Ibid.* 184: 663, 1932.  
III. Etiologic Relationship of Gastric Secretory Defects and Dietary Deficiency to the Hypochromic and Macrocytic Anemias of Pregnancy and the Treatment of These Conditions, *Ibid.* 185: 539, 1933.  
Strauss, M. B.: The Etiology and Treatment of Anemia in Pregnancy, *J. A. M. A.* 102: 281, 1934.  
The Etiology and Prevention of Anemia in Pregnancy, *Ann. Int. Med.* 9: 38, 1935.
- Studdiford, W. E.: Macrocytic Hyperchromic Anemia of Pregnancy. With the Report of a Case and a Review of the Literature, *Am. J. Obst. & Gynec.* 28: 539, 1934.
- Davies, D. T.: Anemia in Pregnancy, *Practitioner* 134: 290, 1935.
- Adair, F. L., Dieckmann, W. J., and Grant, K.: Anemia in Pregnancy, *Am. J. Obst. & Gynec.* 32: 560, 1936.
- Eastman, N. J.: Anemias of Pregnancy, *Internat. Clin.* 2: 257, 1935.
- Dieckmann, W. J., and Wegner, C. R.: Blood in Normal Pregnancy; Blood and Plasma Volumes, *Arch. Int. Med.* 53: 71, 1934.  
Dieckmann, W. J., and Wegner, C. R.: Studies of the Blood in Normal Pregnancy, *Arch. Int. Med.* 53: 188, 1934.
- Ivy, A. C., Morgan, J. E., and Farrell, J. L.: Effects of Total Gastrectomy; Experimental Achylia Gastrica in Dogs With Occurrence of Spontaneous Anemia and Anemia of Pregnancy, *Surg., Gynec. & Obst.* 53: 611, 1931.

- 9 Roslin, P D New Capillary Hemocrit, *Proc Soc Exper Biol & Med* 28 491, 1931
- 10 Hooper, C W Smith, H P, Belt, A I, and Whipple, G H Blood Volume Studies, *Am J Physiol* 51 205, 1920
- 11 Wintrobe, M M, Shumacker, H B, and Schmidt, W J Values for Number, Size and Hemoglobin Content of Erythrocytes in Normal Dogs, Rabbits, and Rats, *Am J Physiol* 114 502, 1936
- 12 Spiegelberg, O, and Gescheider, R Untersuchungen über die Blutmenge trächtiger Hunde, *Arch f Gynak* 4 112, 1872
- 13 Nasse, H Das Blut der Schwangeren *Arch f Gynak* 10 315, 1876
- 14 Schmidt, H R, Bickenback, W, and Jönen, P Stoffwechselphysiologische Untersuchungen an trächtigen Hündinnen mit besonderer Berücksichtigung der chemischen Zusammensetzung der Organe, *Ztschr f Geburtsh u Gynak* 91 527, 1927
- 15 Muller, R B, Dragstedt, C A, and Bradley, J D Hemoglobin Regeneration in Gastrectomized Dogs, *Am J Physiol* 105 443, 1933
- 16 Dragstedt, C A, Bradley, J D and Meid, T B Effect of Iron on Hemoglobin Regeneration in Gastrectomized Dogs, *Proc Soc Exper Biol & Med* 33 58, 1935
- 17 Kellogg, T, Mettier, S R, and Purvance, K Studies on Hypochromic Anemia in Dogs, Evaluation of Standard Bread Diet and of Meat Diet on Formation of Hemoglobin Before and After Gastrectomy, *J Clin Investigation* 5 241, 1936
- 18 Mettier, S R, Kellogg, F and Purvance, K Studies on Hypochromic Anemia in Dogs, Evaluation of Predigested Beef, Iron, and Liver Extract on Formation of Hemoglobin After Gastrectomy, *J Clin Investigation* 16 107, 1937
- 19 Hugouenq, L Recherches sur la statique des elements minéraux et particulièrement du fer chez le fauve humain, *Compt rend Acad d sc* 128 1054, 1890
- 20 Nicholas, J S, and Bosworth, L B Determination of Amount of Hemoglobin Present in Rat Fetuses During Development, *Am J Physiol* 83 400, 1928
- 21 Zeidberg, L D Quantitative Determination of Changes in Hemoglobin Concentration, Volume of Red Cells and Erythrocytes in Blood of Rabbit Fetuses at Various Stages During Last Third of Pregnancy, *Am J Physiol* 90 172, 1929
- 22 Davies, D T, and Shelley, U Some Observations on Hypochromic Anemia and Its Relation to Pregnancy, *Lancet* 227 1094, 1934
- 23 Goodall, J R, and Gottlieb, R Association of Pregnancy, Hypochromic Anemia and Achlorhydria, Preliminary Report, *Canad M A J* 35 50, 1936
- 24 Lyon, E C, Jr Anemia in Late Pregnancy, Preliminary Report, *J A M A* 92 11, 1920
- 25 Fullerton, H W Hypochromic Anemias of Pregnancy and Puerperium, *Brit M J* 2 577, 1936
- 26 Bethell, T H Blood Changes in Normal Pregnancy and Their Relation to Iron and Protein Supplied by Diet, *J A M A* 107 564, 1936
- 27 Mettier, S R, and Minot, G R Effect of Iron on Blood Formation as Influenced by Changing Acidity of Gastrointestinal Contents in Certain Cases of Anemia, *Am J Sc* 181 25, 1931
- 28 Churel, H, Meyer, G M, and Leveue, R A The Influence of the Removal of Fragments of the Gastrointestinal Tract on the Character of Nitrogen Metabolism III The Excision of the Stomach *Am J Physiol* 26 369, 1910
- 29 Emery, E S, Jr Cause of Faulty Digestion in Dogs Without Stomachs, *Am J Digest Dis & Nutrition* 2 599, 1935
- 30 Richter, O, Ivy, A C, and Meyer, A F Study of Dog's Stomach and Liver for Substances Effective in Pernicious Anemia, *Proc Soc Exper Biol & Med* 31 550, 1934
- 31 Ivy, A C, Richter, O, Meyer, A F, and Greengard, H Relation of Gastrectomy to Anemia on Presence of Substances Effective in Pernicious Anemia in Canine Stomach and Liver, *Am J Digest Dis & Nutrition* 1 116, 1934
- 32 Bussabarger, R A, Ivy, A C, and Richter, O To be published
- 33 Sharp, E A, McKern, R M, and Vonder Heide, F C Pernicious Anemia, Behavior of Various Extracts of Stomach and Duodenum Used to Induce Remissions, *Ann Int Med* 4 1282, 1931
- 34 Meulengracht, E Continued Investigations on Presence of Antianemic Factor in Preparation of Dried Stomach Substance From Cardia, Fundus and Pylorus, and Duodenum, Preparations From Duodenum (Preliminary communication), *Acta med Scandinav* 85 79, 1935
- 35 Schemensky, W Zur Pathologie der perniziösen Anämie Therapeutische Erfolge mit Verfütterung getrockneten Schweinedickdarms Mit einem Anhang. Therapeutische Betrachtungen zur Colitis gravis *Ztschr f Klin Med* 128 128, 1935
- Schemensky, W Zur Pathologie der perniziösen Anämie Therapeutische Erfolge mit der Verfütterung getrockneten Schweinedickdarms, *Deutsche med Wochenschr* 61 961, 1935

# TULAREMIA FOLLOWING INJURY WHILE PERFORMING POST-MORTEM EXAMINATION ON HUMAN CASE\*

WITH REPORT OF THE POST-MORTEM FINDINGS IN A CASE OF PULMONIC  
TYPE OF TULAREMIA

J. O. WEILBAECHER, JR., M.D., AND E. S. MOSS, M.D., NEW ORLEANS, LA.

THE sources of infection and the manner of spread of tularemia in man have been summarized by Francis<sup>1</sup> in a recent publication. He lists more than twenty animal and insect hosts capable of conveying the infection, but states that there is no record of transfer of the disease from one human being to another. Having observed an instance of such transfer, we are prompted to report it, and additionally describe the post-mortem findings in a case of the pulmonic type of tularemia.

One of us (E. S. M.) accidentally incised her thumb while performing a post-mortem examination on the body of a negro who, though diagnosed clinically as having had typhoid fever, proved to be a victim of tularemia pneumonia. The development of an ulcer at the site of the accidental wound with associated regional lymphadenopathy suggested the diagnosis of tularemia. This was verified by the subsequent course of the disease and the repeated demonstration in the blood of specific antibodies in titers as high as 1:2560.

## CASE REPORT

W. K., a 26-year-old negro, was admitted to the Charity Hospital of Louisiana, New Orleans, on December 15, 1936. He had been in good health until eight days previous to admission, when he experienced a sudden onset of headache and fever, which persisted with increasing severity. Additionally, he suffered recurrent pain in the right lower abdominal quadrant and on two occasions attacks of epistaxis.

One week after his death, the Hospital's Social Service Department learned that he had been a peddler by trade and that seven days prior to the onset of his illness he had skinned two hampers of wild rabbits.

Physical examination revealed an acutely ill adult negro, with a temperature of 105° F., respiration 24, and radial pulse 102 per minute. Blood pressure was 125 systolic and 85 diastolic. The skin was hot, moist, and elastic. There was no palpable lymphadenopathy. Eyes and ears were free of pathology, while the nares were partially obstructed by clotted blood. The lungs expanded equally and with moderate excursion. No abnormalities in the breath or voice sounds were detectable, and no râles were heard. The heart was normal in size, its sounds of good quality and its rhythm regular. There were no murmurs. Tenderness was elicited in the right lower abdominal quadrant, but there was no muscular rigidity. There were no palpable viscera or masses. Examination of the urine was negative. The blood contained 4,000,000 red blood cells and 8,500 white blood cells per c.mm. The differ-

\*From the Departments of Medicine and Pathology, Louisiana State University School of Medicine and the Charity Hospital of Louisiana, New Orleans.

Received for publication, January 29, 1938.

ential leucocyte count revealed 60 per cent polymorphonuclear neutrophils, 30 per cent small lymphocytes, and 10 per cent large lymphocytes and monocytes. No malarial parasites were found.

The demonstration in the blood of agglutinins for *Eberthella typhi*, in a titer of 1:320, seemed to corroborate the clinical impression of typhoid fever and treatment for such was instituted. During the ten days he remained in the hospital, his condition grew steadily worse and he died December 25, 1936, at 9 p.m.

### POST MORTEM EXAMINATION

**Body** Post mortem examination was made ten hours after death. Inspection of the body revealed a well developed, well nourished, young colored male. Body heat was absent. Rigor mortis was present. There were no notable gross lesions or abnormalities.

**Peritoneal Cavity** There was no free fluid or adhesions present and the membranes were smooth, moist, and glistening.

**Pleural Cavities** No adhesions were present. Both cavities were filled with slightly cloudy straw colored fluid. The right visceral pleura was covered with patches of yellowish fibrinous exudate and a considerable number of fibrinous strands were free in the fluid. The serosa of the left cavity was free of exudate.

**Pericardial Cavity** The cavity contained the usual amount of clear, straw colored fluid and the serosa was smooth and glistening.

**Heart** The heart weighed 350 gm. The myocardium was reddish in color and firm in consistency. The valves presented no abnormalities and the endocardium appeared normal. The coronary arteries were patent.

**Lungs** The right lung weighed 1,225 gm, the left lung 900 gm. The serosa of both lungs were grayish pink in color with scattered yellowish areas measuring 1 to 2 cm in diameter. Areas of patchy consolidation could be felt throughout all lobes, but the lower right lobe was uniformly firm in consistency. On section there was diffuse consolidation of this latter area with scattered areas of necrosis. All other lobes showed scattered patches of consolidation and necrosis.

**Liver** The liver weighed 2,175 gm. The anterior edge was smooth, rounded, and extended 6 cm below the right costal margin. Glisson's capsule was smooth and normal in color. On section the liver markings proved indistinct, and the surfaces revealed were pale red in color.

**Spleen** The spleen weighed 160 gm. It was dark red in color and very soft in consistency. The pulp scraped with ease.

**Pancreas, Adrenals, and Gastrointestinal Tract** There were no notable pathologic changes.

**Kidneys** The right kidney weighed 210 gm, the left kidney weighed 220 gm. The capsules stripped with ease leaving smooth pink surfaces. On cut section the line of demarcation between cortex and medulla was distinct. The cortical zone was slightly widened.

### MICROSCOPIC EXAMINATION

**Lung** The sections obtained from the consolidated portions (Fig 1) showed large areas resembling caseous necrosis with interspersed groups of alveolar sacs filled with exudate. The necrotic areas were composed of either a mixture of polymorphonuclear leucocytes, macrophages, fibrin, and necrotic debris or a granular bluish staining mass of necrotic material in which only a few cellular elements were discernible. In the non necrotic areas the alveolar septal cells were swollen. The exudate was variable, consisting either of a network of fibrin in which there were scattered polymorphonuclear leucocytes, red blood cells and macrophages, or a mixture of polymorphonuclear leucocytes and macrophages. The exact composition of the exudate in any given place varied between these two extremes. The cytoplasm of the majority of the macrophages was vacuolated or uniformly pale staining (foam cells) but many contained phagocytosed cells or cellular fragments particularly nuclear fragments. Some of the latter cells measured as much as 20 micra in diameter. No multinucleated giant cells were observed. Although both the arteries and veins in the con-

## DISCUSSION

Increased atmospheric pressure and increased percentage of oxygen resulted in growths predominantly subsurface. This was greater in the pressure chamber where the oxygen tension was approximately twice as great. In most instances subsurface growth was good and growth weights would have to be taken to determine the comparative amounts with those of surface growths.

When the surface and subsurface tendencies on the various media and under the various tensions are averaged, that on glutamic acid medium in 99.5 per cent oxygen is comparatively good and has the most subsurface.

When seventeen-day growths are removed from the chambers and growth occurs, it is the type growth that would have occurred if the tubes had been placed in the atmosphere originally.

Growths become more surface in 1 per cent oxygen with no observable decrease in growth on glutamic acid medium, but with marked decrease in growth on Sabouraud's medium where growths appear to be trying to leave the surface and may prove dead. In the latter instance the optimum oxygen tension for growth is absent.

In the atmosphere Sabouraud's medium and 4 per cent peptone, 1 per cent dextrose medium show similar amounts of growth and similar surface tendencies. Media with 0.25 per cent peptone, 4 per cent dextrose, and 1 per cent peptone, about one-tenth the surface tendency and four-fifths the growth of Sabouraud's medium. Other amino acids and hair hydrolyzates show like tendencies to glutamic acid. Pig skin hydrolyzate shows one-half the subsurface tendency of glutamic acid.<sup>3-6</sup> Glutamic acid medium with 0.5 per cent sodium lactate and/or 0.5 per cent sodium phosphate shows about one-half the surface tendency and three-fourths the growth on Sabouraud's medium.<sup>7</sup> The latter effect seems confined to certain organisms.

Factors of importance in growth of organisms may be summarized as follows: Selectivity with reference to the organism is dependent on the atmosphere-medium relationship. The medium is characterized by its constituents, physical and chemical state, and the atmosphere by gas tensions, humidity, temperature, and air velocity. Organisms are characterized by their evolutionary status, adaptations, and resistances. In disease the organism, necessary atmosphere-medium relationship, together with tissue injury, are necessary. Such injury to tissue differentiates pathogenic from saprophytic fungi. In disease selectivity is determined by the constituents of the organ and its oxygen tension. It is questionable that the atmosphere ever functions in a strictly passive sense.

The atmosphere-medium relationship is equally important with bacteria which usually possess no power of invasion of medium and must be studied in shake culture. Increase in oxygen tension alone increases depth of growth, increase in pressure by nitrogen having no such effect.

Skin lesions in guinea pigs at high altitudes due to *Achorion gypseum*<sup>8</sup> have been observed to have a longer incubation period, to be more superficial and less extensive; this has been correlated with clinical findings in tuberculosis. The latter disease is interesting because of extensive work<sup>9</sup> on the gas relations of the tubercle bacillus.

## SUMMARY

The atmosphere medium relationship determines amount, type of growth, and surface or subsurface tendencies of microorganisms

Invasion of medium results from the organism seeking the most favorable atmospheric medium relationship for growth and departure of growth. Subsurface tendency apparently bears a proportionality to the anaerobic nature of the organism.

Inhibition of growth claimed by other workers<sup>10</sup> for high oxygen tensions should be decided only after growth weight studies.

Partial oxygen tension determines the depth and presence of growth on a specific medium.

Sparse superficial or no growth on Sabouraud's medium under 1 per cent oxygen often results in death of the organism.

Invasion of tissues and selectivity for tissues in disease are undoubtedly dependent on the medium (tissue) atmosphere (oxygen tension) relationship and pathogenicity on the added injury through the organism. Simple factors may change this relationship entirely.

Peptone dextrose agar from Difco Laboratories Inc.  
 d glutamic acid from Hoffman LaRoche  
 Nitrogen Airco  
 Oxygen Airco 99.5 per cent

## REFERENCES

- 1 Williams, J. W. Bacterial Growth Spectrum Analysis. I. Methods and Applications, *Am J M Technol* 4: 58, 1928.  
 Bacterial Growth "Spectrums" II. Their Significance in Pathology and Bacteriology, *J Path & Bact* In Press.  
 Factors of Importance in Susceptibility to Disease. *Urol & Cutan Rev* In Press.
- 2 Rosenthal, L. "Chromium Sulphuric Acid" Method for Anaerobic Cultures, *J Bact* 34: 317, 1937.
- 3 Williams, J. W. Invasiveness of Skin Infections Caused by Pathogenic Fungi and Subsurface Mycelium. *Science* 83: 396, 1936.
- 4 Williams, J. W. The Possible Importance of Mediums Causing Surface and Subsurface Growth of Pathogenic Fungi to the Diagnosis and Treatment of Disease, *J Lab & Clin Med* 22: 268, 1936.
- 5 Williams, J. W. Allergy, Immunity and Growth of Pathogenic Fungi in Vivo and in Vitro, *Urol & Cut Rev* 41: 117, 1937.
- 6 Williams, J. W. Subsurface Growth of Pathogenic Fungi on Peptone, Hair, Pig Skin and Cysteine Cystine Mediums, *Arch Dermat & Syph* 36: 581, 1937.
- 7 Williams, J. W. Factors of Importance in the Production of Surface Growth of Pathogenic Fungi on Culture Mediums, *Arch Dermat & Syph* To be published.
- 8 Kadisch, B. E., and Loewy, A. The Course of Infection With *Achorion gypseum* in High Altitudes, *Arch f Dermat u Syph* 163: 149, 1931.
- 9 Novy, F. G., and Soule, M. H. Microbic Respiration. II. Respiration of the Tubercle Bacillus, *J Infect Dis* 36: 168, 1925.
- 10 Karsner, H. T., and Saplar, O. Influence of High Partial Pressures of Oxygen on the Growth of Certain Molds, *J Infect Dis* 39: 231, 1926.

## THE PHYSIOLOGIC RESPONSE OF PLEURAL SURFACES TO IMPLANTED DUSTS\*

WILLIAM R. BRADLEY, M.S., AND MELVIN W. FIRST, B.S., DETROIT, MICH.

LATELY several inquiries have sought to establish the physiologic responses of various body tissues to divers dusts in different particle size ranges. As a consequence, there is at hand extensive experimental information dealing with the action of many specific dusts and mixed dusts, some of which have been applied in studies under conditions simulating the exposures experienced by workers in dusty trades. Among others, Mavrogordato, Gardner, and Gye, respectively, have studied the action of dusts in the experimental animal by the inhalation method. In this procedure, it appeared necessary that artificial dusting be continued over a long period, such as from two to five years. Obviously, the time factor hampers inquiries carried out in this manner.

As early as 1924, Sayers,<sup>1</sup> in an effort to reduce the time period required for the appraisal of the properties of unknown dusts, began experiments to determine the action of divers dusts injected into the peritoneal cavity of guinea pigs. He concluded that living animal tissue in any portion of the body tends to respond in essentially the same manner to any dust, but that the action for every individual dust was specific. Selecting the peritoneal cavity as the most suitable point of application, he observed that, in addition to the foreign body reaction provoked by all dusts, highly different changes resulted from the presence of specific dusts. Thus, quartz leads to extensive proliferation of fibrous tissue, but this was not elicited by limestone or coal. In reports based upon continuation of these experiments, Sayers and his associates<sup>2-4</sup> described a technique for the determination of the fibrogenic properties of unknown dusts following deposition within the peritoneal cavity. In addition to the usual foreign body reaction, three general types of specific reaction were described, namely, the proliferative, the absorptive, and the inert. Within ninety days the type of reaction provoked may be determined, and often changes provoked within thirty days may provide proper conclusions.

These three reactions in more detail were found to be:

*The proliferative response:* characterized by the production of nodules that progressively increased in size. These nodules, when occurring in clumps, fused together and formed a large single mass. Numerous capillaries were present on the surface and throughout the nodules. The appearance was that of cellular proliferation. It was their premise that this pathology was appar-

\*From the Bureau of Industrial Hygiene, Department of Health, Detroit.  
Received for publication, February 3, 1938.

ently due to the chemical irritation supplied by the solution of silica in tissue. In ninety days the nodules become more firm, contracted, and fibrous in appearance.

The *inert reaction* obtained when the injected dust was neither absorbed nor initiated a cellular proliferation. The nodules become more flattened and spreading with time. Fine particles of dust were usually earned over rather extensive areas by phagocytes.

The *absorptive response* showed the formation of irregular distinct nodules which were often clumped. These nodules became progressively smaller in size as the interval between injection and examination increased, until the original dust eventually disappeared leaving a small area of fine particles at the site of the nodules. In time these also disappeared without the formation of scar tissue.

Thus a reaction was produced in terms of weeks instead of years as was the case in dusting chamber inhalation experiments. The results of these intraperitoneal injection experiments, especially in connection with siliceous dusts, are in conformity with what would be expected from a knowledge of the clinical picture of the actions of these dusts in the pulmonary tissues of human beings.

McCord and his associates,<sup>5,8</sup> in a large series of experiments, supported findings of Miller and Savers and added two modified types of responses, namely (1) a *mixed response*, produced by mixed dusts containing some free silica characterized by a reaction which was predominantly inert, but which had a few typical silicotic nodules scattered throughout the tissue, and (2) a *miliary proliferative response*, produced by dusts that possessed more irritant properties than those exhibiting the uncomplicated proliferative reaction. When such dusts were introduced it appeared that their irritant component excited such marked movement of the organs that the dusts were widely distributed throughout the peritoneal cavity. Wherever particles of this type of dust were deposited, a proliferative response was obtained.

This virtual biologic assay has yielded an abundance of helpful information regarding the harmful effects to be expected from a variety of dusts.

The effect on tissues elsewhere in the body following the introduction of dusts forms the basis for a number of publications. Kettle<sup>9</sup> studied the responses of subcutaneous tissues, and Kettle and Hilton<sup>10</sup> introduced dusts directly into the lungs through the trachea. Their conclusion from this latter study was that the reaction of the lung to dust introduced in this method is essentially the same as to dust introduced by the inhalation method and may advantageously replace the dusting chamber for many purposes for which it has hitherto been considered necessary. Polcoid<sup>11,12</sup> used the cornea and conjunctiva in his studies. Drinker and his associates<sup>13</sup> and Stuber<sup>14</sup> have injected senescent and industrial dusts containing free silica into the lymphatics of dogs. It was their opinion that the cellular response of the draining lymph nodes might be developed into a rapid means of determining the toxicity of certain dusts.



## THE PHYSIOLOGIC RESPONSE OF PLEURAL SURFACES TO IMPLANTED DUSTS\*

WILLIAM R. BRADLEY, M.S., AND MELVIN W. FIRST, B.S., DETROIT, MICH.

LATELY several inquiries have sought to establish the physiologic responses of various body tissues to divers dusts in different particle size ranges. As a consequence, there is at hand extensive experimental information dealing with the action of many specific dusts and mixed dusts, some of which have been applied in studies under conditions simulating the exposures experienced by workers in dusty trades. Among others, Mavrogordato, Gardner, and Gye, respectively, have studied the action of dusts in the experimental animal by the inhalation method. In this procedure, it appeared necessary that artificial dusting be continued over a long period, such as from two to five years. Obviously, the time factor hampers inquiries carried out in this manner.

As early as 1924, Sayers,<sup>1</sup> in an effort to reduce the time period required for the appraisal of the properties of unknown dusts, began experiments to determine the action of divers dusts injected into the peritoneal cavity of guinea pigs. He concluded that living animal tissue in any portion of the body tends to respond in essentially the same manner to any dust, but that the action for every individual dust was specific. Selecting the peritoneal cavity as the most suitable point of application, he observed that, in addition to the foreign body reaction provoked by all dusts, highly different changes resulted from the presence of specific dusts. Thus, quartz leads to extensive proliferation of fibrous tissue, but this was not elicited by limestone or coal. In reports based upon continuation of these experiments, Sayers and his associates<sup>2-4</sup> described a technique for the determination of the fibrogenic properties of unknown dusts following deposition within the peritoneal cavity. In addition to the usual foreign body reaction, three general types of specific reaction were described, namely, the proliferative, the absorptive, and the inert. Within ninety days the type of reaction provoked may be determined, and often changes provoked within thirty days may provide proper conclusions.

These three reactions in more detail were found to be:

*The proliferative response:* characterized by the production of nodules that progressively increased in size. These nodules, when occurring in clumps, fused together and formed a large single mass. Numerous capillaries were present on the surface and throughout the nodules. The appearance was that of cellular proliferation. It was their premise that this pathology was appar-

\*From the Bureau of Industrial Hygiene, Department of Health, Detroit.  
Received for publication, February 3, 1938.

## THE PRESENT WORK

In the present series of experiments 50 rabbits were used. They received varying doses from 1 to 4 c.c. of a dust suspension (1 c.c. of suspension equals 0.1 gm. dust). Ten received amorphous silica;\* ten, white rouge;† ten, calcium carbonate;‡ and ten received magnetite.§ The remainder were not injected and used as control animals. The majority of animals were submitted to autopsy thirty days after injection. Twenty animals were autopsied, as a control measure, at periods of fifteen, forty-five, sixty and ninety days. The injected material containing as much as 0.4 gm. of dust did not result in any apparent discomfort to the animals either immediately after injection or for the duration of the experiment. The weights of the animals over the experimental period showed normal gains and infection or injury produced no loss of animals.

*Preparation of the Dusts.*—Four dusts, shown by intraperitoneal injections into guinea pigs<sup>4, 8</sup> to be representative of the three main types of reactions elicited by dusts in animal bodies, were chosen for this experiment: white rouge and amorphous silica as proliferative dusts, magnetite as an inert dust, and calcium carbonate as an absorptive dust.

The dusts were injected in the form of suspensions, prepared by placing 10 gm. of dust in 100 c.c. of isotonic saline solution and shaking thoroughly.

*Technique of the Injection.*—No preliminary preparation of the animal was found necessary. The rabbit was stretched out by his front and rear legs on a table with the side to be injected uppermost. A 5 c.c. hypodermic syringe with a glass plunger and an 18 gauge needle were used to inject the suspensions. With particles of the size used, little difficulty was experienced due to locking of the syringe.

The pleural cavity was entered by introducing the needle through the skin in the fourth intercostal space about 3 cm. lateral to the sternum, directing the needle downward and slightly backward and carefully inserting the point until it barely penetrated the parietal pleura and entered the free pleural space. When certain that the needle was in the free space and not in the deep muscles or chest organs, the suspension of dust was injected. As a precaution against the possibility of entering the lung tissue, it was found advantageous to introduce a few cubic centimeters of air into the pleural space before injecting the material, thereby producing a partial pneumothorax.

\*Amorphous silica—an air floated cryptocrystalline material, specific gravity 2.6, 100 per cent through 600 mesh. Contains 98.47 per cent free silica ( $\text{SiO}_2$ ). Purchased from Fredrick B Stevens Co., Detroit. Size distribution of particles—25 per cent under 1 micron, 75 per cent under 2 microns, 95 per cent under 5 microns, 99 per cent under 10 microns.

†White rouge—air floated from "Silica #00" mined in Southern Illinois. Contains 99 per cent free silica ( $\text{SiO}_2$ ), 100 per cent through 600 mesh. Purchased from Terminal Sales Corp., Detroit. Size distribution of particles—15 per cent under 1 micron, 62 per cent under 2 microns, 86 per cent under 5 microns, 96 per cent under 10 microns.

‡Calcium carbonate—a Mallinckrodt analytical reagent, more than 99 per cent calcium carbonate, mechanically screened through a 325 mesh screen. Size distribution of particles—10 per cent under 1 micron, 20 per cent under 2 microns, 46 per cent under 5 microns, 77 per cent under 10 microns.

§Magnetite or black iron oxide (ferrous-ferrie)—a Mallinckrodt reagent mechanically screened through a 325 mesh screen. Size distribution of particles—25 per cent under 1 micron, 55 per cent under 2 microns, 75 per cent under 5 microns, 87 per cent under 10 microns.

*Gross Pathology.*—White rouge and amorphous silica, composed almost completely of free silica, produced identical gross reactions in the pleural cavity of the rabbit. The siliceous material was scattered throughout the cavity, although the larger deposits were found at the site of injection on the chest wall, and on the pericardium. Numerous small deposits were found on the lungs and on the mediastinal pleura overlying the vena cava. Nodules were rarely found on the diaphragm, which is worthy of note since in intraperitoneal injections the diaphragm is one of the tissues more frequently affected. This picture of the distribution of dust in the pleural space was true for black iron oxide and calcium carbonate as well as for the silica dusts.

*White rouge and amorphous silica* (Fig. 1) produced nodules that were comparatively large in size and showed a strong tendency to fuse with those adjacent. Deposits as large as 2 cm. in diameter, adhering to the pleura and



Fig. 1.—Free silica (white rouge). Large irregular masses on pleural surface of chest wall characteristic of a proliferative reaction.

pericardium, were commonly seen after thirty days. Numerous capillaries were present on the surfaces and throughout the nodules, giving the appearance of cellular proliferation. In almost every case, thirty or more days after injection, numerous adhesions between chest wall and lung, chest wall and pericardium, pericardium and lung, and lung and diaphragm were observed.

*Calcium carbonate* produced a reaction in the rabbit chest that has been designated as absorptive. Forty-five days after injection the material had completely disappeared from the body and to all appearances had left the chest uninjured. After thirty days, two of the rabbits showed a single, small, round, hard, white deposit of calcium carbonate loosely adhering to the pleural surfaces by one or two fine strands of fatty connective tissue. Capillaries were not present in or on the deposits and there were no evidences of cellular proliferation.

*Magnetite* (Fig. 2) tended to aggregate into one large flat smooth glistening deposit, either at the site of injection or on the pericardium. Occasional small deposits were seen on the visceral pleura. Ninety days after injection the quantity of magnetite in the pleural cavity approximated the amount injected. No cellular proliferation was observed. Thus, since neither a diminution of material nor cellular proliferation was in evidence, the reaction was termed inert.

Whenever animals were dispatched during early days after the injection of any dust, there were observable the usual and expected evidences of a foreign body reaction. Prior to the expiration of thirty days, the evidences of the initial foreign body reaction had completely subsided.



Fig. 2.—Black iron oxide (magnetite). Masses of pigmented material on pericardial pleura. An inert reaction.

*Microscopic Pathology.*—*White rouge* (Fig. 3). Microscopically the tissue response is the proliferative reaction characteristic of free silica. There are large masses of cells having the appearance of macrophages in which can be seen particulate foreign material. In many areas the nuclei are more or less distinctly grouped in rosettes around collections of particles of foreign material. In many of these nuclear groups the boundaries between the nuclei are indistinct or not visible, giving these masses the appearance of aggregation giant cells. In a few areas two types of change are observed in the nuclear rosettes. One change consists of an intensification of staining, an increase in opacity of the cytoplasmic material, and a decrease in cytoplasm relative to the amount of nuclear material present. The other change ob-

served consists of an increase in the cytoplasm in proportion to the amount of the nuclear material with the cytoplasm becoming paler. Here the nuclei are small and are placed in the extreme periphery of the cell mass or aggregate. In a few cell groups where these cell processes are most marked, a nuclear fragmentation occurs. These changes seem to represent progressive stages of degeneration. Fibroblasts and young fibrous tissue cells are present in varying numbers in different sections of the material and also in different portions of the same section.

*Magnetite* (Fig. 4). Microscopically the tissue response is the so-called inert reaction. Here large amounts of deeply pigmented particulate material appear in the sections. Most of this occurs intracellularly and has the ap-



Fig. 3.—Free silica (white rouge). Photomicrograph (high power). Section from nodule shown in Fig. 1. A proliferative lesion with giant cell formation and particulate foreign material in surrounding cells.

pearance of being phagocytized in such large quantities that the details of the phagocytic cells are obscured. A delicate connective tissue meshwork with a few fine capillaries supports the phagocytic cells. There are also present a few large angular masses of foreign material and around these are typical foreign body giant cells.

*Calcium carbonate.* Microscopically no characteristic changes are apparent.

*Multiple Injections in the Same Animal.*—It was carefully noted that in no case was there a spread of material from the injected side of the chest to either the opposite pleural space or to the peritoneal cavity. This suggested, in the interest of economy, the utilization of one animal for the testing of three dusts simultaneously, injections being made into each pleural cavity and into the peritoneal cavity.

A second way in which the three body cavities of one animal may be employed is to use a single dust but to make the three injections at such intervals that at autopsy it will be possible to observe the resultant pathology fifteen, thirty, and sixty days after injection.

In this study ten of the experimental animals were submitted to the implantation of dusts into both pleural spaces and in one case dust was introduced into all three of the sites suggested above. In no case did the animals show any ill effects from the multiple injections.

*X Rays*—X rays were made of the chests of five rabbits to determine whether the characteristic proliferative nodule produced by free silica could be distinguished from nonproliferative nodules by this means. Two of the five rabbits had received white rouge, two amorphous silica, and one magnetite.

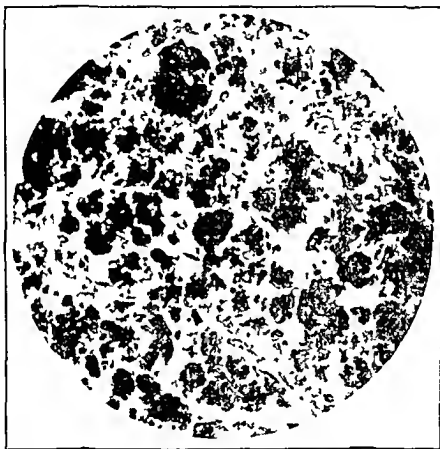


Fig. 4—Black iron oxide (magnetite). Photomicrograph (high power). Section from pigmented mass shown in Fig. 2. An inert reaction.

Definite shadows could be seen on the x-ray plates of the magnetite rabbit and on one of the white rouge rabbits. The location of these shadows was subsequently correlated with the autopsy findings, but it was concluded that although the nodules could be readily seen on the x-ray plate, they were of no diagnostic significance.

#### SUMMARY

Forty rabbits were subjected to intrapleural implantation of dust and, with ten additional rabbits as controls, were autopsied after fifteen, thirty, forty five, sixty and ninety days.

Typical inert, absorptive and proliferative reactions were produced by the pleura in response to various dusts.

These responses were essentially the same as produced in serous cavities elsewhere in the body by other workers.

The injection of a dust or several dusts into both pleural cavities and into the peritoneal cavity of the same animal was found to be a practical procedure resulting in the saving of animals and time.

The pleural cavity, due to its close proximity to lung tissue, proved to be a suitable location for the study of the physiologic response of serous surfaces to implanted dusts.

The authors extend thanks to W. L. Brosius, M.D., Pathologist, Department of Health, Detroit, for microscopic examinations of tissues and preparation of statements in this report dealing with microscopic changes.

#### REFERENCES

1. Sayers, R. R.: Health Hazards in the Mining Industry, U. S. Bureau of Mines, Report of Investigation, No. 2660, 1924.
2. Miller, J. W., and Sayers, R. R.: The Physiological Response of the Peritoneal Tissue to Dusts Introduced as Foreign Bodies, U. S. Pub. Health Rep. 49: 80, 1934.
3. Miller, J. W., and Sayers, R. R.: Microscopic Appearance of Experimentally Produced Dust Nodules in the Peritoneum, U. S. Pub. Health Rep. 50: 1619, 1935.
4. Miller, J. W., and Sayers, R. R.: The Physiological Response of the Peritoneal Tissue to Certain Industrial and Pure Mineral Dusts, U. S. Pub. Health Rep. 51: 1677, 1936.
5. McCord, Carey P., Fleming, Richard L., Ainslee, Harriet, and Johnston, Jan: The Measurement of the Harmfulness of Dusts for Humans Through the Agency of Animal Reactions, Surg. Gynec. & Obst. 63: 120, 1935.
6. McCord, Carey P.: Tripoli as a Source of Silicosis, J. A. M. A. 106: 913, 1936.
7. McCord, Carey P., Ainslee, Harriet, Johnston, Jan., and Fleming, Richard L.: The Action of Silica as Modified by the Presence of Alkalies, Indust. Med. 5: 17, 1936.
8. McCord, Carey P., Kasper, J. A., and Brosius, W. L.: A Biologic Test for the Determination of the Fibrogenetic Properties of Dust, Ohio State M. J. 33: 394, 1937.
9. Kettle, E. H.: The Interstitial Reactions Caused by Various Dusts and Their Influence on Tuberculous Infections, J. Path. & Bact. 35: 395, 1932.
10. Kettle, E. H., and Hilton, R.: The Technique of Experimental Pneumoconiosis, Lancet 1: 1190, 1932.
11. Policard, A., and Mouriquand, V.: Methode de detection et d'étude des particules minerales dans les tissus specialement le tissu pulmonaire, Bull. d'histol. appliq. à la physiol. 7: 193, 1930.
12. Policard, A., and Rollet, J.: Reactions du tissu corneen vis-a-vis des particules minerales siliceuses, Bull. d'histol. appliq. à la physiol. 8: 52, 1931.
13. Drinker, C. K., Field, M. F., and Drinker, P.: The Cellular Response of Lymph Nodes to Suspensions of Crystalline Silica and to Two Varieties of Sericite Introduced Through Lymphatics, J. Indust. Hyg. 16: 296, 1934.
14. Stuber, Katharina: The Cellular Response of Lymph Nodes to Various Dust Suspensions Introduced Into Lymphatics, J. Ind. Hyg. 16: 282, 1934.
15. Higgins, G. M., and Lemon, W. S.: Absorption From the Pleural Cavity of Dogs. I. The Cytologic Aspect, Am. J. M. Sc. 181: 697, 1931.
16. Merklen, P., Waitz, R., and Kabaker, J.: Les Injections d'encre de Chine dans les Epauchements Plcurax Humains, Compt. rend. Soc. de biol. 110: 308, 1932.
17. Arnaud, J., Chadourne, P., and Weiller, R.: Action de la créosote dans les lavages de plèvre. Rev. de la tuberc. 2: 1192, 1936.
18. Desbaillet, E.: Contribution à l'histophysiologie de la plèvre, Arch. internat. de méd. expér. 3: 315, 1927.
19. Bratiano, S., and Bratiano, C.: Absorption des graisses animales par la plèvre, Compt. rend. Soc. de biol. 98: 461, 1928.
20. Matsuda, Tsuneo: J. Gastroenterol. Japan 2: 1003, 1927.
21. Brodsky, J. F.: Physiologie und Pathologie der Resorption aus der Pleurahohle, Ztschr. f. d. ges. exper. Med. 61: 24, 1928.
22. Bethune, N.: Pleural Poudrage: New Technic for Deliberate Production of Pleural Adhesions as Preliminary to Lobectomy, J. Thoracic Surgery 4: 251, 1935.

## SEASONAL AND REGIONAL FACTORS IN ACUTE RHEUMATIC FEVER AND RHEUMATIC HEART DISEASE\*

CLARENCE A. MILES, M.D., CINCINNATI, OHIO

MEDICAL science has made little headway in its attack on the problems of rheumatic infections and the more chronic forms of arthritis, although the recent increase in investigative interest gives rise to a degree of hope. These ailments probably rank close after respiratory infections as disabling factors in the productive activities of human existence. And they have been just as resistant as has the common cold to investigative success. There is, in fact, a more significant relationship between rheumatic infections and those of the upper respiratory tract than this similarity in resistance to investigative success. A great many of the initial rheumatic fever attacks are actually ushered in by acute upper respiratory infection, and both show the same seasonal relationship to storminess. Close study by a number of investigators, however, has failed to provide conclusive proof that the respiratory infections are direct exciting causes of the rheumatic attacks, even though the two may often be closely associated in their seasonal and regional timing.

It is well, therefore, to look closely into all possible factors that may produce regional or time differences in the disease incidence or severity, in the hope that useful etiologic or therapeutic suggestions may show forth. Hence, in this article will be set forth certain significant relationships in the regional and time distribution of rheumatic afflictions. The evidence presented indicates that some factor concerned with the movement of major storm areas over the earth's surface exerts a dominant influence in determining the incidence of these attacks. Let us look briefly at this evidence.

Rheumatic infections, and the resulting disabilities, are perhaps more directly and completely conditioned by climatic and weather characteristics than are any other diseased states. Complaints of this type are infrequent among residents of the tropics or subtropics, and increase rather steadily as one passes outward into the temperate zone storm belts. Rheumatic fever attacks and rheumatic heart disease occur ten to twenty times more often in the worst areas of the storm zones than in the calm warmth of the tropics. And in northern countries the difference in incidence between winter high and summer low is almost as striking as is the regional one. At Cincinnati acute rheumatic fever attacks are over six times as frequent in March as in August and September, while at Buenos Aires little seasonal variation attends the more equitable year round climate. Then, too, those afflicted in the north usually show improvement in symptoms on migration to the climatic calm and warmth of sub-tropical regions.

\*From the Department of Internal Medicine University of Cincinnati  
Received for publication February 16 1938



In Fig. 1 is presented the seasonal distribution of acute rheumatic fever attacks in three widely separated hospitals, one in Cincinnati, another in San Francisco,<sup>1</sup> and the third in Peiping, China.<sup>2</sup> Each curve shows the marked increase in attacks that comes with winter cold and storms, and the great diminution in summer warmth. The accompanying curve of temperature variability due to the passage of storm areas over northern United States is seen to parallel the monthly incidence of rheumatic attacks at Cincinnati quite closely, just as we found for the onset of symptoms of pulmonary tuberculosis at this same city. At Peiping the difference in incidence between winter and summer is slightly less marked than at Cincinnati, and at San Francisco the winter peak is only four times the summer low, while at Cincinnati the attacks are six and four-tenths times more prevalent in late winter. This lesser seasonal swing at San Francisco may well be causally related to the lower incidence of winter storm

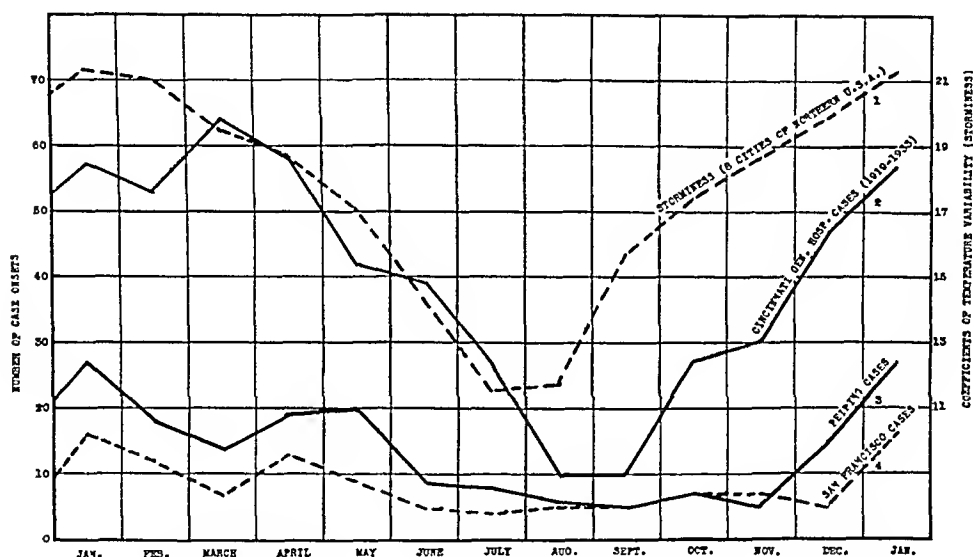


Fig. 1.—Seasonal fluctuation in frequency of acute rheumatic fever attacks.

fluctuations there, for winter and spring weather at Cincinnati is much more subject to abrupt and marked change than is that of San Francisco or a similar latitude in North China.

Regional differences in the incidence of *rheumatic heart disease* have been best estimated by careful examinations of groups of school children. Nichol<sup>3</sup> found only one-fourth as much such heart disease among 1,500 native school children of Miami, Fla., as in a similar number who had migrated to Miami from northern states. Even more marked differences were found by Paul<sup>4</sup> on examination of Indian school children, as shown by the data below:

LOCATION OF GROUPS	LATITUDE	NUMBER EXAMINED	PER CENT WITH RHEUMATIC HEART DISEASE
Montana and Wyoming	44-46°	688	4.5
Northern Arizona and New Mexico	36-37°	1,106	1.9
Southern Arizona	32-33°	1,019	0.5

The same investigator<sup>2</sup> found a 2.2 per cent incidence among 2,624 school children in and around New Haven, Conn., at a latitude between that of the northern and middle groups of Indian children.

Among hospitalized populations there is evident an even greater difference in incidence of *rheumatic heart disease*, estimates of its prevalence in the south ranging from one-twentieth to one-tenth that found in northern hospitals.<sup>3</sup> Fankner and White<sup>4</sup> showed that this finding had world-wide application, for hospitals of middle temperate regions usually had ten to twenty times as great a percentage of their patients so afflicted as was to be found in hospitals of the tropics or subtropics. Coburn<sup>5</sup> found very little rheumatic involvement in Puerto Rico or other tropical countries, although Puerto Ricans migrating to New York had a relatively high frequency of rheumatic infections. There would

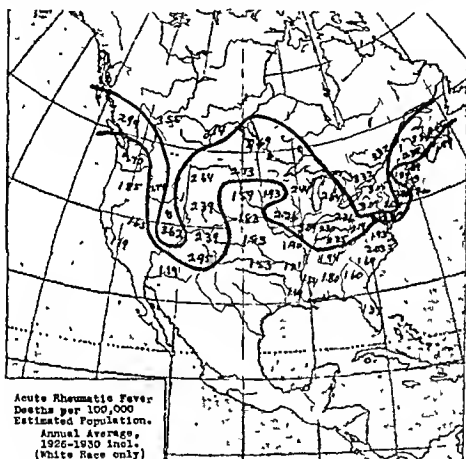


Fig 2 —Regional variations in acute rheumatic fever death rate

seem, then, to be little doubt that rheumatic heart involvement is decidedly more common in the cooler, more stormy portions of the temperature zone than in tropical or subtropical warmth.

Similar differences in the incidence of *acute rheumatic fever* have been found among hospital populations north and south,<sup>6</sup> although no survey of incidence has yet been made in nonhospitalized populations. In Fig. 2 is presented the distribution of acute rheumatic fever *deaths* per 100,000 population (white race only) for a five-year period (1926-1930), showing a much less striking difference in *deaths* from the disease than in its *incidence* among the living. The death rate in the two worst areas is only about twice as high as it is in the least afflicted regions. The Rocky Mountain and higher plateau states to the east form one area of high death rates from this cause, while from Iowa east-

ward to the Atlantic Coast is located a second unfavorable region. A decided latitude gradient is also in evidence in the figures for the white population as given in Fig. 2, but negro death rates afford more striking evidence in this respect.

ACUTE RHEUMATIC FEVER DEATH RATES PER 100,000 COLORED POPULATION  
(1926-1930)

Maryland	7.73	Kentucky	8.31
Virginia	6.39	Tennessee	5.23
North Carolina	4.53	Alabama	3.74
South Carolina	4.25	Arkansas	2.87
Georgia	3.73	Oklahoma	2.76
Florida	3.23	Mississippi	2.54
		Louisiana	2.19

Negro rates are in every state higher than those for the white population, and the increase northward is also more rapid, as has been found to hold with many other climatically conditioned diseases. The latitude gradient is quite evident, however, with both white and colored populations. It can scarcely be related to sunlight intensity, because of the relatively low death rates found well upward along the Pacific Coast and in part of New England. Even in the belt of lower rates up the Plains states the northward increase is seen. No explanation can be offered for the lower death rate in the Plains states, but in this connection it is worthy of note that Faulkner and White<sup>6</sup> cited a remarkably low incidence of the disease and its heart complications in hospital populations of Omaha. This city, the only one cited in the Plains region, recorded a hospital incidence far below that of other cities of similar latitudes.

The reason for the general discrepancy between acute rheumatic fever *deaths* and the *incidence* of the disease and its heart complications in living populations is probably to be found in a more vigorous body resistance to the infection and higher survival rate in the more energetic northerners. This difference in ability to fight such infections as acute appendicitis and tuberculosis has been pointed out<sup>8</sup> as probably due to the generally higher metabolic level and higher vitality of people living in the northern states, as compared to those living nearer the Gulf of Mexico. Such a difference explains quite satisfactorily the finding of a smaller increase in acute rheumatic fever *deaths* toward the north than is found for the *incidence* of the disease in school children and hospital populations. Deaths increase approximately twofold in the worst regions as compared to the regions of low rates, while a tenfold difference in incidence is found. Ability to fight the infection must, therefore, be several times higher in the north than in the south, just as was demonstrated for acute appendicitis and tuberculosis.

In further support of these differences in the disease between our northern and southern states is a seasonal difference described by Greenwood and Thompson<sup>9</sup> for London, Young<sup>10</sup> for England and Wales, Christie<sup>1</sup> for San Francisco, Dieuaide and Chang<sup>2</sup> for Peiping, and Faulkner and White<sup>6</sup> for eastern United States. In Fig. 1 have been set forth the seasonal variations in acute rheumatic

fever attacks at Cincinnati, San Francisco, and Peking, China. Coburn describes a somewhat similar seasonal distribution of acute rheumatic admissions to northern hospitals. He states that at Buenos Aires little seasonal difference in attack frequency exists, winter being only slightly higher than summer. Thus again points to weather instability as a dominant factor, particularly when associated with cold or body chilling from exposure. Storminess at Buenos Aires is greatest in their summer season, the winters being cool but calm. Swift<sup>11</sup> also cites several studies relating the frequency of attacks quite closely to periods or seasons of changeable weather.

Based on these definite regional and seasonal differences in the disease various groups of rheumatic heart disease patients have been sent from northern regions to tropical warmth, and with good results. While in the calm warmth of Miami or the West Indies, northernborn patients experience fewer recurrences and show improvement generally. Such improvement is soon lost however, when they must again experience the northern winter and early spring weather. For lasting benefit they must forego living in the stormy north and take up permanent residence in subtropical warmth. Due to the rarity of storm changes in the Southwest, residence there near the Mexican border would probably be found even more beneficial than in southern Florida or the West Indies, where storms of the tropical hurricane type so frequently come. There is a strong suggestion that the sudden change of barometric pressure which accompanies tropical hurricanes, may be just as important a factor in promoting many types of infectious attacks as are similar pressure changes accompanying temperate zone storms. In temperate regions we have been prone to place more blame on temperature and humidity fluctuations in explaining the evil effects of sudden storm changes, but further research may well show pressure change to be another responsible factor. Rentschler and co-workers<sup>12</sup> found closer correlation of arthritic pain attacks with storm changes in barometric pressure than with other meteorologic factors.

Mention should be made of the severity of acute rheumatic infections in one subtropical region of relatively low storminess. In the mining region of South Africa rheumatic infections seem to be as severe as in any of the stormy temperate climates. Whether this severity is related to work in the deep mines cannot be said until the question has been closely investigated. Very high respiratory disease death rates were observed among these miners until air conditioning in the mines lessened the temperature differences between their depths and the outside air (and, of course, also aided in cleansing the air in the mines). Close study of the rheumatic problem in the South African region might provide information of great value in regard to the general biology of these afflictions.

The more even seasonal distribution of rheumatic fever attacks between summer and winter in Buenos Aires, reported by Coburn,<sup>7</sup> is of some significance in establishing the etiologic importance of atmospheric instability, especially when such storminess is associated with lower temperatures. Abrupt temperature changes seldom strike southern hemisphere inhabitants during the winter season. In the north, on the other hand, we not only have frequent and abrupt

cold waves, but they usually are ushered in with precipitation to enhance the chances for body chilling. Young<sup>10</sup> showed for England and Wales that rheumatic fever attacks bore a definite relationship to precipitation. He also found, as did Paul and associates<sup>5</sup> that the attacks were most frequent among the poor, *who are likely to have least effective protection against weather changes in their dwellings or clothing* (italics and suggestion mine). A critical observer cannot but be impressed by the frequency with which hospital histories record body wetting or chilling just preceding the rheumatic attack. The frequency of acute respiratory infections also seems to play a definite part in the initiation of the rheumatic attacks, but they too are initiated largely by weather changes. By and large, then, we must consider rheumatic fever and its complications directly or indirectly conditioned by the climate and the weather. Proper protection from sudden weather changes should go far to reduce the likelihood of rheumatic attacks.

*But for the individual already afflicted, such protection is usually inadequate and migration out of the storm belts is advised.* Where economic status permits, those afflicted with rheumatic heart disease (or chorea) should be taken south to avoid the cold and storminess of the winter and early spring season. If possible, permanent change of residence is still more preferable, *the choice region from a climatic standpoint in this country being the southern parts of New Mexico, Arizona, and California.* For the great number of afflicted children whose parents cannot afford such a change, a good suggestion would be that convalescent camps or farms be established by the government in choice spots of the Southwest where the best possible chance for recovery, or an existence of limited usefulness, may be allowed. The total burden on public funds would probably be no more than is required by their frequent and prolonged stays in northern hospitals at high overhead costs. Such convalescent units in the Southwest could well serve for patients from the North with a variety of afflictions (chronic sinusitis, chronic bronchitis, arthritis, and tuberculosis) as well as the rheumatic heart patients. Those suffering from overstimulation or metabolic exhaustion in the North (patients with diabetes, pernicious anemia, hyperthyroidism and early hypertensive tendencies, or heart failure) would also do much better by similar change of residence, although their region of first choice should perhaps be in the moist heat of the Gulf states.

Since the disabling character of rheumatic afflictions in the North makes the care of such patients a heavy burden on their families or public institutions, it would really seem a wise public health move for the federal government to sponsor these health camps. Such a move would involve recognition of a new principle in public health activities, since the presence of these diseases in a population group entails no known health threat to persons not afflicted, with no call for segregation. But why should not public attention be given to the care of the afflicted themselves? The indicated treatment being change of residence to a distant state, sponsorship of such a program must devolve on federal government agencies. Many details of organization and operation of this move-

ment would need careful study, but the obvious benefit to the mass of afflicted individuals warrants the effort.

In recent years considerable numbers of northern rheumatic patients have been sent to Florida for successive winter seasons, and with rather disappointing results. Recrudescences have occurred during such southern sojourns, and the general course of the disease has not been markedly altered in many cases. On the basis of storminess, or weather variability, this result is rather to be expected. Winter in Florida has almost as great a degree of weather variability as does northern winter, although at a much higher mean temperature level. One should not expect, then, much help to rheumatic patients in a southern sojourn for the winter months only. The stagnant summer warmth is the more beneficial time. The Southwest, on the other hand, shares very little in the winter storminess that afflicts the remainder of the country. The following data illustrate well the futility of migration to Florida for the winter months alone in an effort to escape storm effects.

COEFFICIENTS OF TEMPERATURE VARIABILITY

STATIONS USED	JAN	FEB	MARCH	APRIL	MAY	JUNE	JULY	AUG	SEPT	OCT	NOV	DEC
Boston, New York, Cincinnati, St. Louis, Chicago, Minneapolis, Cleveland, 1871-1932	21.5	21.0	19.5	18.7	17.1	14.5	11.5	11.8	15.7	17.4	18.7	19.9
Pampa, Raleigh, Houston, El Paso 1926-1928	19.9	14.6	17.0	15.7	12.0	11.1	7.0	5.3	11.0	14.4	14.7	18.2

In summary, then, it seems safe to say that in acute rheumatic fever we have an infection much similar to those of the respiratory tract in its climatic conditioning. It strikes most frequently in the cooler, more stormy portions of the temperate zones and is there a more violent form of infection. In the tropics and subtropics it is markedly less frequent and more benign in course. The survival rate, judging roughly from the data at hand, seems to be considerably higher in the more vigorous people of the north, just as has also been found true in tuberculosis and acute appendicitis attacks. Acute rheumatic fever strikes most frequently in winter and early spring months and seldom in summer warmth. Children with rheumatic heart disease also do best in summer warmth and suffer more recrudescences during winter and early spring. Since the heart damage is permanent and recrudescence so common in northern cold and storms, it is definitely indicated that these unfortunate patients be taken to a subtropical climate where the calm warmth will allow the disease to take a more benign course, and where the lowered general metabolic level will lessen the work load on the damaged heart muscle. Migration for such patients should be to regions well south, preferably near the Mexican border from El Paso west, rather than to southern Florida or the West Indies where tropical storms and long debilitating summer heat might mitigate against chances for complete quiescence of the disease process. Migration should be permanent, and not for the winter alone.

## REFERENCES

1. Christie, Amos: Rheumatic Fever in Northern California, *Am. Heart J.* 12: 153, 1936.
2. Chang, F. C., and Dieuaide, F. R.: A Clinical Study of Rheumatic Fever, *Chinese M. J.* 51: 581, 1937.
3. Nichol, E. Sterling: Geographic Distribution of Rheumatic Fever and Rheumatic Heart Disease in the United States, *J. LAB. & CLIN. MED.* 21: 588, 1936.
4. Paul, John R.: Rheumatic Heart Disease Among American Indian School Children in Different Climates, *The Milbank Memorial Fund Quarterly* 15: 135, 1937.
5. Paul, John R., Harrison, E. R., Salinger, R., and DeForest, G. K.: The Social Incidence of Rheumatic Heart Disease, *Am. J. M. Sc.* 188: 301, 1934.
6. Faulkner, James M., and White, Paul D.: The Incidence of Rheumatic Fever, Chorea and Rheumatic Heart Disease, *J. A. M. A.* 83: 425, 1924.
7. Coburn, A. F.: *The Factor of Infection in the Rheumatic State*, Baltimore, 1931, Williams & Wilkins Co.
8. Mills, C. A.: Acute Appendicitis and the Weather, *J. Med.* 15: 30, 1934; Susceptibility to Tuberculosis: Race or Energy Level? *Am. J. M. Sc.* 189: 330, 1935.
9. Greenwood, M., and Thompson, T.: On Meteorological Factors in the Aetiology of Acute Rheumatism, *J. Hyg.* 7: 171, 1908.
10. Young, Mathew: A Preliminary Study of the Epidemiology of Rheumatic Fever, *J. Hyg.* 20: 248, 1921.
11. Swift, Homer F.: *Rheumatic Fever*, Nelson Loose-Leaf Living Medicine, New York, 1928, Thomas Nelson & Sons, Chapter XIV, Vol. I.
12. Jones, T. Duckett, White, Paul D., Roche, C. F., Perdne, Jean Jones, and Ryan, H. A.: The Transportation of Rheumatic Fever Patients to a Sub-tropical Climate, *Proc. Sixth Conference on Rheumatic Diseases*, Atlantic City, June 7, 1937, *J. A. M. A.* 109: 1309, 1937.
13. Rentschler, E. B., Van Zant, F. R., and Rowntree, L. G.: Arthritic Pain in Relation to Weather Changes, *J. A. M. A.* 92: 1895, 1929.

## REPORT OF A CASE OF ESSENTIAL HYPERTENSION OF MORE THAN TWENTY-FIVE YEARS' DURATION SHOWING NO RENAL ARTERIOLAR CHANGES AT AUTOPSY\*

SHEPARD SHAPIRO, M.D., NEW YORK, N. Y.

THE question still remains: Does the renal arteriolar sclerosis of essential hypertension, which is said to be a constant accompaniment of this disease, precede the rise in vascular tension?

Experimentally it has been demonstrated that renal ischemia causes hypertension. On this basis there is the possibility that in essential hypertension renal arteriolar sclerosis impairs the blood supply to the kidneys, giving rise to a state of chronic ischemia which in turn induces the sustained elevation in the blood pressure.

Goldblatt<sup>1</sup> and his co-workers have demonstrated that by constricting the renal artery they have been able to produce hypertension. They showed that a state of renal ischemia resulted and that in response to this a pressor substance was liberated by the kidney and poured into the circulation through the renal vein. These findings have been corroborated and have enjoyed general acceptance.

\*From the medical services of the Hospital for Joint Diseases, Dr. A. A. Epstein, Chief, New York.

Received for publication, February 25, 1938.

However, the application of these experimental findings to cases of essential hypertension requires further elucidation. The hypothesis already mentioned assumes that the arteriolar sclerosis is the initial disturbance which causes the augmented blood pressure.

The purpose of this paper is to report an illustrative case pertinent to this question. The patient was a known hypertensive for more than twenty-five years, and at the autopsy showed normal kidneys and normal arterioles.

### CASE HISTORY

T. R., first consulted me in December, 1928. She was then 43 years old. When she was 22 years of age, it was discovered that she had high blood pressure. When she was 25, she developed exophthalmos. A diagnosis of Graves' disease, in addition to hypertension, was made and a subtotal thyroidectomy was performed. Her blood pressure at that time was 220/110. Following this there was no abatement in either the exophthalmos or the hypertension. Eleven years after the first operation, she was again operated on for recurrence of the symptoms of Graves' disease. At this time the blood pressure was still elevated 210/100.

She consulted me in December, 1928, complaining chiefly of shortness of breath, especially after exertion.

Her weight was 199 pounds (90.4 kg.). The obesity, however, was symmetrically distributed throughout the body. There were no indications of myxedema. The lungs were clear throughout. The heart was enlarged to the left. The rhythm was regular. A systolic blow was audible at the apex. The second aortic sound was accentuated and ringing in quality. The blood pressure was 225/120. The liver and spleen were not palpable and no masses were felt. The urine contained no sugar or albumin, or other abnormal constituents. The eyes were equally exophthalmic and showed no retinal pathology. The basal metabolic rate was plus 4 per cent.

She was seen again in April, 1929, at which time her weight was 200 pounds (90.9 kg.) despite dietary restriction, and the blood pressure 200/100. The basal metabolic rate was minus 2 per cent.

She was examined at irregular intervals thereafter, averaging about twice a year until April, 1933. During this period her condition showed no noteworthy change. The weight remained at about 200 pounds (90.9 kg.) and the blood pressure 200/220 systolic and 100/110 diastolic. The urine was consistently free of abnormal constituents. The specific gravity of occasional specimens was 1.024 and 1.020. The basal metabolic rate remained within normal limits. Orthodiagnostic examinations indicated enlargement of the left ventricle.

In January, 1935, she complained of indefinite precordial pains, especially after exertion. The blood pressure was 190/100. Examination of the heart revealed no striking change. An electrocardiogram showed inversion of the T wave in Lead I and left axis deviation. The urine was negative. The blood urea nitrogen was 20.4 mg. and sugar 98 mg. per 100 c.c. blood. The weight was 185 pounds (84 kg.).

In September she complained of epigastric distress, especially after eating. She had lost 10 pounds (4.5 kg.) in weight and had observed some increase in her dyspnea after exertion. Examination revealed a drop in blood pressure to 170/90. The soft blowing systolic murmur had become more pronounced and occasional extrasystoles were heard. The liver was not palpable, but for the first time the spleen was felt. A barium series done shortly after this showed a constant defect along the greater curvature in the midportion of the stomach. The enlarged spleen was clearly visualized. There was no obstruction or delayed emptying of the stomach or any part of the gastrointestinal tract. The possibility of carcinoma of the stomach with extension to the gastrosplenic omentum and invasion of the spleen was considered remote.

On March 26, 1936, the patient was suddenly seized with an attack of dizziness, following which she vomited fresh blood. The hematemesis recurred, and she had several tarry bowel movements. She was removed to the Hospital for Joint Diseases, where in spite of all efforts including two direct transfusions, she died of exsanguination four days later. An autopsy was performed one hour post mortem by Dr. C. J. Sutro.



*Autopsy Findings.*—Only the pertinent findings are herein recorded:

“Heart: The organ is in its normal anatomical site. The pericardial sac contains a small amount of clear fluid. The lining is everywhere smooth and glistening. The heart weighs 300 grams. Most of the enlargement is due to hypertrophy of the left heart. The right auricle and ventricle are somewhat larger than normal. Their endocardial linings are everywhere smooth and glistening. The tricuspid and pulmonary valves show nothing very striking. The cavity of the right auricle and ventricle is only a little larger than normal. The myocardium of the left ventricle measures in places from 2 to 3 cm. The endocardial lining of the left heart is everywhere smooth and glistening. The papillary muscles of the left ventricle are hypertrophied to almost twice the normal size. The chordae tendineae are shortened. The mitral and aortic valves present nothing very striking. There is a small amount of calcification at the aortic ring. The commissures are normal in appearance. The mouths of the coronary arteries are patent. The sections of these vessels show only scattered atheromatous plaques. There is no stenosis present.

“Microscopic section shows myocardial degeneration; slight coronary sclerosis without narrowing.

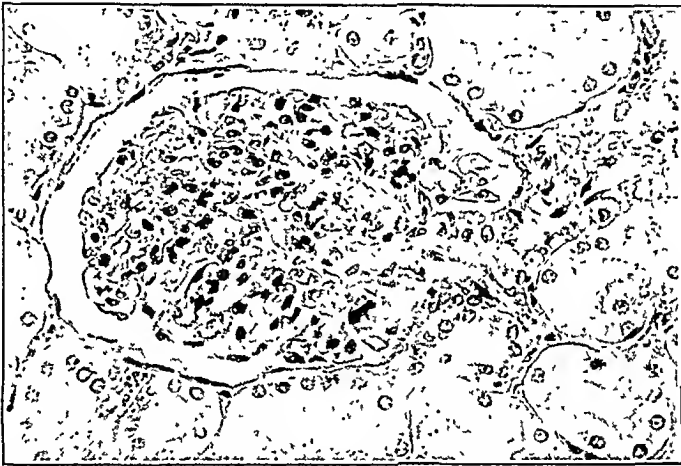


Fig. 1.—High power. Illustrating typical architecture of both kidneys. Normal glomeruli and arterioles.

“Lungs: A few thin fibrous adhesions are present in the right and left pleural cavities. Otherwise there are no abnormalities.

“Vascular System: The aorta is elastic throughout. Its arch is dilated to a very slight extent. An atheromatous plaque attached to one end of the intima is seen at the summit of the arch of the aorta. The thoracic and lumbar portions show a few atheromatous plaques with occasional calcification.

“Liver: The organ is in its normal anatomical site. It weighs 3,000 grams. Its free margin is two fingers below the ninth rib in the right mid-clavicular line. The liver is brown-yellow in color. The capsule of Glisson is uniformly wrinkled; beneath it can be seen closely set brown to yellow nodules. These measure from 2-5 mm. in diameter. The entire liver is composed of these colored small nodules. The caudate and quadrate lobes are also involved by a similar pathologic process. Here too, are present small yellowish nodules. The liver is firm to the touch. Cross-section shows the presence of the above described nodules. No large mounds of liver tissue are to be seen. The hepatic ducts in the liver show no closure at any point.

“Microscopic sections show normal architecture completely replaced by pseudobubbles of varying size, separated by connective tissue which is infiltrated by many small round cells and an occasional polymorphonuclear leucocyte. The liver cells show degeneration with conspicuous vacuolization.

"Gastro Intestinal Tract The esophagus is in its normal anatomical site. Its mucosa is discolored reddish and numerous recent clots are seen on it. The stomach is of average size. The entire mucosa is red in color. Large clots are seen in the lumen of the stomach. The rugae are somewhat atrophic. Along the lesser curvature is seen an ulceration measuring  $2\frac{1}{2}$  cm in diameter. The tissue about the ulceration is markedly thickened so that the wall of the stomach measures from  $\frac{1}{2}$  to  $\frac{3}{4}$  of 1 cm in thickness. No vessels are seen in or about the ulceration. No perforation is found. The duodenum shows nothing very striking. Large blood clots are seen in the jejunum and ileum. The mucosa of the small intestine shows nothing very striking except for reddish discoloration. The large intestines also contain clots.

"Microscopic section through the edge of the gastric lesion shows typical peptic ulcer.

"Pancreas Shows nothing abnormal.

"Spleen The organ is in its normal anatomical site. It weighs 460 grams. Part of the capsule is thickened. This presents itself as a scaling surface. Section shows indistinct Malpighian corpuscles. The organ is firm to the touch. It has a deep brown color. There is an increase in stroma. The pulp is firm. The vessels show nothing striking.

"Microscopic sections show perisplenitis with hyalinization and considerable iron pigment deposition, sinus hyperplasia with some fibrosis of the pulp.

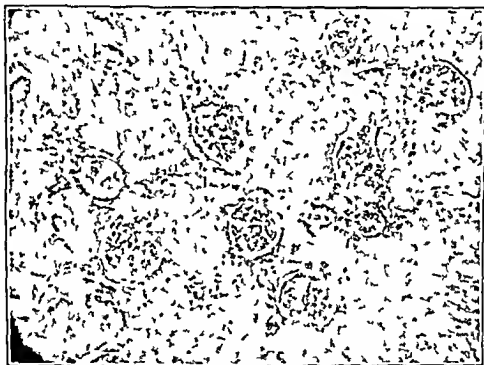


Fig 2—Low power. Same as Fig 1.

"Adrenals Both adrenals are in their normal anatomical sites. They are of average size and shape. Sections show that their cortices contain a large amount of yellow material.

"Kidneys Both kidneys are of average size and shape. They are normally situated. The perirenal fat is normal in quantity and has an orange color. The capsules strip with ease. Section shows that the cortices are normal in width. The cortico medullary junctions are sharp. The calyces are normal in size. The pelves show nothing very striking. The ureters are average width. The mucosae are everywhere smooth and glistening.

"Microscopic section shows no significant change." (Figs 1 and 2)

#### SUMMARY

The patient had hypertension for more than a score of years, she succumbed at the age of 50 to an apparently independent condition. At autopsy the heart weighed 300 gm and the kidneys on gross and microscopic section were free of any abnormality characteristic of hypertension. In addition the patient had had Graves' disease necessitating partial thyroidectomy at the age of 30 and again at 41, which was evidently a superimposed condition and not a cause of

the hypertension, because the blood pressure was known to be elevated several years before symptoms of hyperthyroidism appeared and continued markedly elevated for many years after thyroidectomy, during which interval the metabolic rate was consistently normal.

A gastric ulcer had been present and healed spontaneously with very few symptoms during life to indicate its existence. The patient also had cirrhosis of the liver. This was probably a sequela to the hepatic degeneration which has been found to occur in Graves' disease and the splenomegaly in turn was secondary to this.<sup>2</sup>

#### DISCUSSION

It appears that cases, such as the one herein described, are rare. A few have been referred to in the literature.<sup>3</sup> However, the fact that they do occur is of the greatest importance. They establish the independence in at least certain cases of hypertension and renal impairment.

There is no proof here that the arteriolar sclerosis which constantly accompanies essential hypertension<sup>4</sup> is the result of the high blood pressure. To argue whether this patient would eventually have developed arteriolar thickening would be speculation. The findings, however, in the case herein reported indicate that essential hypertension may arise and continue independently of any renal impairment.

#### CONCLUSIONS

A case of essential hypertension of more than twenty-five years' duration complicated by recurrent Graves' disease is reported.

Evidences of renal impairment were consistently absent.

At autopsy the kidneys showed no sclerosis of the arterioles or other pathology.

This case establishes the possibility that normal arterioles including those of the kidneys are not incompatible with hypertension of many years' duration.

This case indicates further that essential hypertension may arise and continue from causes still unknown but independent of any functional or organic renal pathology.

#### REFERENCES

1. Goldblatt, Harry, Lyneh, James, Hanzal, R. F., and Summerville, W. W.: Studies on Experimental Hypertension: I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia, *J. Exper. Med.* 59: 347, 1934.
2. Marine, D., and Lenhart, C. H.: Pathological Anatomy of Exophthalmic Goiter; the Anatomical and Physiological Relations of the Thyroid Gland to the Disease; the Treatment, *Arch. Int. Med.* 8: 265, 1911.
3. Bell, E. T., and Clawson, B. J.: Primary (Essential) Hypertension, *Arch. Path.* 5: 939, 1928.  
Fishberg, A. M.: Hypertension and Nephritis, Philadelphia, 1930, Lea & Febiger.
4. Fishberg, A. M.: Anatomical Findings in Essential Hypertension, *Arch. Int. Med.* 35: 650, 1925.

# THE EFFECT OF LIVER EXTRACT UPON POLYCYTHEMIA VERA\*

RALPH H. MAJOR, M.D., KANSAS CITY, KAN.

THE demonstration by Minot and Murphy<sup>4</sup> of a liver fraction, effective in the treatment of pernicious anemia, has focused attention upon this disease as a deficiency disease. The proof that this anemia is apparently due to the deficiency of a certain factor quite logically led to the theory that polycythemia vera is a disease in which this factor is present in excess. This theory was supported by Morris, Schiff, and Foulger<sup>7</sup> and by Briggs and Oerting,<sup>2</sup> who described improvement in the blood count in patients of polycythemia following gastric lavage. This has also been supported by Patton, Allardyce, and McKeown,<sup>7</sup> who describe polycythemia vera as the antithesis of pernicious anemia. More recently Baráth and Fulop<sup>1</sup> have described an increase in the amount of the hemopoietic factor in the gastric juice of patients with polycythemia vera as compared with normal individuals.

A number of years ago it occurred to us that if polycythemia vera were due to an excess of a hemopoietic, antianemic factor, then treatment of such patients with liver extract would further increase the blood count of patients with polycythemia vera. Two observations were made in 1931 and a third observation was made in 1936. The blood counts were carried out in the usual manner, and the hemoglobin estimations were carried out with the Haden-Hausser hemoglobinometer. A brief summary of these patients is as follows:

CASE 1.—G. P., No 33448, male, aged 65 years, was admitted to the hospital November 21, 1930, and was dismissed February 13, 1931. This patient on admission showed a red cell count of 8,930,000, white cell count of 11,550, and hemoglobin of 110 per cent. Five days after admission the patient was placed on Lilly's liver extract, No 343, two capsules three times a day. This therapy was continued until the patient's dismissal from the hospital at which time his red cell count was 6,660,000.

	R. B. C.	W. B. C.	HEMOGLOBIN	RETICULOCYTES (PER CENT)
11/21/30		11,550	110	
11/22/30	8,930,000			
11/23/30	8,620,000	13,650	112	
11/30/30				0.8
12/ 1/30	7,360,000	12,050	109	11
12/ 2/30	6,790,000			None
12/ 4/30	7,030,000			0.14
12/ 5/30	6,660,000			0.26
12/ 6/30	7,050,000			
12/ 8/30	7,420,000	11,400	105	

\*From the Department of Internal Medicine, University of Kansas School of Medicine, Kansas City.

Received for publication, March 4, 1938.

## CASE 1.—CONT'D

	R.B.C.	W.B.C.	HEMOGLOBIN	RETICULOCYTES (PER CENT)
12/ 9/30	7,630,000			
12/10/30	7,720,000			None
12/11/30	7,500,000			None
12/12/30	7,100,000			None
12/13/30	6,540,000			None
12/15/30	7,100,000			None
12/16/30	7,200,000			
12/17/30	6,840,000	11,800	111	None
12/18/30	8,280,000			
12/19/30				None
12/27/30	7,560,000			
12/30/30	7,540,000			
1/ 2/31	6,000,000			
1/ 6/31	6,370,000			
1/ 7/31	6,440,000			None
1/ 8/31	6,620,000			None
1/ 9/31	6,300,000			None
1/12/31	5,760,000	10,100	104	
1/13/31	5,400,000			None
1/14/31	5,740,000			
1/15/31	5,650,000			
1/16/31	5,600,000			
1/19/31	5,740,000			None
1/20/31	5,570,000			None
1/21/31	6,300,000			None
1/22/31	5,840,000			None
1/31/31	6,720,000	11,000	106	
2/ 6/31	6,430,000			
2/ 7/31	6,540,000			
2/11/31	6,660,000	10,200	105	

CASE 2.—L. F., No. 33926, female, aged 56 years, was admitted to the hospital January 12, 1931, and was dismissed January 14, 1931. This patient was admitted to the hospital following a severe epistaxis at which time the cell count was red blood cells 6,350,000, white blood cells 21,200, and hemoglobin 97 per cent. This patient was placed on liver extract, No. 343, two capsules three times a day, on January 26, 1931, and the treatment continued until January 9, 1933. The blood picture showed the following findings:

	R.B.C.	W.B.C.	HEMOGLOBIN
1/21/31	6,170,000	18,000	87
1/28/31	6,910,000	22,500	88
2/ 1/31	7,900,000	19,400	103
2/11/31	6,710,000	15,450	90
4/15/31	7,350,000	18,800	97
5/ 6/31	8,090,000	18,650	100
6/ 3/31	8,200,000	18,200	103
7/21/31	8,410,000	17,850	97
8/18/31	7,400,000	1,900	94
9/23/31	8,120,000	18,850	100
10/21/31	8,630,000	19,450	100
11/15/31	6,900,000	12,400	100 (15.5 gm.)
11/25/31	8,950,000	17,200	103
12/ 2/31	8,760,000	17,900	104
12/ 9/31	8,380,000	18,350	103
12/23/31	9,020,000	14,350	103
1/ 6/32	8,450,000	21,300	97
2/ 3/32	7,700,000	8,900	90
3/23/32	7,940,000	12,250	94 (14.5 gm.)
4/15/32	9,090,000	12,000	97
1/ 9/33	7,640,000	16,100	103

CASE 3—J L, No 69390, male, aged 55 years, was admitted to the hospital November 9, 1937, and was dismissed November 30, 1937. This patient was admitted to the hospital showing a blood picture of RBC 8,960,000, WBC 10,200, and hemoglobin 148 per cent (23 gm). This patient was given a highly potent liver extract in the form of Reticulogen, 0.5 cc, and these injections were given every other day. The blood counts were as follows:

	RBC	WBC	HEMOGLOBIN	RETICULOCYTES (PER CENT)
11/ 8/37	8,960,000	10,200	148	
11/11/37	9,200,000	7,500	154	2.5
11/12/37	8,840,000	12,350	154	2
11/13/37	9,000,000	12,000	154	2.2
11/15/37	8,700,000	13,200	153	1
11/16/37	9,010,000	13,700	158	1.85
11/17/37	8,990,000	14,500	160	1.54
11/18/37	9,100,000	10,400	145	0.66
11/19/37	8,890,000	12,000	156	1.2
11/20/37	8,340,000	12,200	154	1
11/22/37	4,820,000	12,400	150	0.6
11/23/37	8,200,000	12,800	154	0.3
11/24/37	8,500,000	12,100	150	0.6
11/26/37	8,900,000	12,200	154	1.3
11/27/37	9,020,000	12,700	148	0.8
11/29/37	8,000,000	11,900	146	0.4
11/30/37	7,950,000	13,750	152	1

The above findings were interesting to us in view of the previous publications of Stephan,<sup>8</sup> Ogawa,<sup>6</sup> and Hitzenger.<sup>3</sup> These authors described a very satisfactory fall in the red blood cell count of patients with polycythemia vera following treatment with liver extract. Indeed, this treatment is recommended by them for polycythemia vera. Their views being, at least theoretically, the antithesis of those previously cited. Our observations have led us to conclude that the liver extract is without any specific effect on the red blood cell count, the hemoglobin or the reticulocyte count in patients suffering from polycythemia vera. Such variations as we saw, we felt were only the variations untreated patients often show and bore no relationship to treatment with liver extract.

#### REFERENCES

- 1 Baráth Eugen, and Fulop Josef. Untersuchungen über den pathogenetischen Zusammenhang von perniziöser Anämie und splenomegaler Polycythämie, *Ztschr f klin Med* 129 172 1935
- 2 Briggs John Francis, and Oerting Harry. Influence of Gastric Lavage on Familial and Non familial Erythremia, *Minnesota Med* 18 499, 1935
- 3 Hitzenger, Karl. Zur Frage der Pathogenese der Polycythaemia vera, *Ztschr f klin Med* 129 778, 1936
- 4 Minot, George R, and Murphy, William P. Clinical Observations of Patients With Pernicious Anemia Partaking of a Special Diet, *Tr A Am Physicians* 41 72 1926
- 5 Morris Roger S, Schiff, Leon and Foulger, Margaret. Erythremia (Polycythemia Vera)—A Theory Regarding Etiology, *J Med* 13 318, 1932
- 6 Ogawa Juntaro. Über den Einfluss der Lebersubstanz auf Polycythaemia vera, zugleich ein Beitrag zur Milzdiatherapie, *Jap J M Sc Tr VIII, Int Med, Pediat & Psychiat* 2 267, 1932
- 7 Patton, William D, Allardyce, John, and McKeown, Thomas. Is Polycythemia Vera the Antithesis of Pernicious Anemia? *Canad M A J* 27 502, 1932
- 8 Stephan, Richard. Symptomatische Polyglobulie und Lebersubstanz Therapie, *Klin Wchnschr* 9 1068, 1930

## THE REDUCTION OF PRONTOSIL SOLUBLE BY URINE\*

JOHN V. SCUDI, NEW YORK, N. Y.

IT WAS observed that, upon standing, urine specimens from patients being treated with prontosil frequently decolorized the dye they contained. Investigation revealed that upon standing for more than one day normal urine specimens, collected at random under nonsterile conditions, were capable of decolorizing appreciable amounts of dyestuff. Addition of phenol or mineral acid stopped the reduction process, as did boiling the urine for five minutes prior to the addition of the dye. Two urine specimens taken by catheter did not decolorize sterile dye solutions. It has been suggested<sup>1</sup> that the in vivo activation of prontosil may result from bacterial reduction processes. In the present instance, bacterial intervention functions mainly in increasing the urinary pH by alkaline fermentation. Cultures of *B. coli* did not decolorize very dilute sterile dye solutions. Addition of ammonium hydroxide, sodium hydroxide, or sodium carbonate accelerated the decolorization process.

When added to normal urine in concentrations of 15 to 25 mg. per cent, prontosil was consistently reduced if 25 c.c. of the urine was boiled for fifteen to thirty minutes in the presence of sufficient alkali to darken the hue of the prontosil. A wide variety of urine samples was studied. In general, normal urine was not capable of decolorizing concentrations of the dye above 25 to 30 mg. per cent under these conditions. This value varied somewhat with different samples, but more markedly with the conditions under which the reduction was carried out. Under these conditions uric acid, creatinine, cevitic acid, and cysteine did not reduce the dye. However, glucose which normally occurs in urine in concentrations up to 0.4 per cent is known to reduce azo dyes in alkaline media. In agreement with the assumption that glucose is the reducing substance active in normal urine, it was observed that urine from diabetic patients decolorized higher concentrations of the dye, and similarly, adding glucose to the urine produced reduction of increased amounts of the dyestuff. Other reducing sugars, fructose, lactose, maltose, etc., produced similar reductions.

Air oxidation impedes the reduction process. Standard solutions of glucose (1.0M and 0.1M) were titrated from a pipette into a casserole containing 10 c.c. of boiling standard prontosil solution and 1 c.c. of sodium hydroxide (1 per cent). For high concentrations of the dye, the glucose was added in one or two molar amounts, and the solution was boiled for three minutes after each addition. For lower concentrations ten moles of glucose were added at a time. Dye concentrations of 0.0001M and 0.0005M required 200

\*From the Department of Pathology, Harlem Hospital, New York, Dr. S. Weintraub, Director.

Received for publication, March 14, 1938.

to 250 moles of glucose, 0.001M required 70 to 90 moles, and 0.01M dye required only 17 to 20 moles of glucose for complete decolorization. A similar influence of air oxidation in the urinary reduction of the dye at room temperatures was evident: surface layers of the urine remained red much longer than the lower layers.

Attempts to standardize the use of prontosil for the determination of urinary sugar were discontinued since unsatisfactory end points were obtained. The final color depends upon the concentrations of both reactants and the pH. Alkaline solutions of glucose become colored from a yellow to a deep red brown, and this color varies directly with the glucose concentration and is darkest in alkaline media. The resultant aminonaphthol is readily air oxidized in alkaline medium to a quinonoid green color, which varies in intensity directly with the dye concentration, the time of heating, the extent of aeration, and the pH. A similar color change was observed in the reductions of the dye by normal urine which was allowed to remain at room temperatures. After complete reduction of the dye, aeration produced a greenish cast in the specimens, indicating the absence of hydrazines.

It has been shown that prontosil<sup>2</sup> and similar dyes<sup>3</sup> are reduced in the animal. Reduction may also occur in urine. Complete recovery of the injected dye in excretion studies necessitates that these reduction processes be considered.

#### REFERENCES

1. Long, P., and Bliss, E.: P-Aminobenzenesulfanamide and Its Derivatives. Experimental and Clinical Observations on Their Uses in the Treatment of Beta Hemolytic Streptococci Infections, *J. A. M. A.* 108: 32, 1937.
2. Fuller, A. T.: Is P-Aminobenzenesulfanamide the Active Agent in Prontosil Therapy? *Lancet* 1: 194, 1937.
3. Sisley, P., and Porcher, C.: Du sort des matières colorantes dans l'organisme animal, *Compt. rend. Acad. d. sc.* 152: 1063, 1911.



# LABORATORY METHODS

---

## CULTURE VERSUS GUINEA PIG INOCULATION IN THE DIAGNOSIS OF TUBERCULOSIS\*

---

WILLIS A. MURPHY, M.D., AND DOROTHY RHOADES DUERSCHNER, A.B.,  
NEW YORK, N. Y.

---

IT WAS not until Dorset's description of his egg medium in 1902 that attention was focused upon the potentialities of cultural methods for the successful isolation of tubercle bacilli from contaminated material. It is beyond the scope of this paper to discuss all the media and their various modifications that have been brought forth since that time. Suffice it to say that the techniques of Petroff,<sup>1</sup> Corper,<sup>2</sup> Sweany and Evanoff,<sup>3</sup> Lowenstein,<sup>4</sup> and Petragani,<sup>5</sup> among others have proved satisfactory in the hands of some investigators and unsatisfactory in others.

Herman, Hansman and De Capito,<sup>6</sup> using Corper's medium, studied a large series of 537 cases with both guinea pig inoculations and cultural methods. Of these, 48 proved positive by both methods. In an additional 28 cases the guinea pig alone became positive, whereas in 7 instances the culture alone became positive. Naturally, they concluded that animal inoculation was a more reliable procedure. Nevertheless, upon analyzing their cases, one notes that guinea pig fatalities accounted for twice as many failures as culture tube contaminations, drying of media, and breakage. The authors did not state whether they divided the material equally between the animal and the culture tubes.

In 1931 Stadnichenko and Sweany<sup>7</sup> compared the results of both methods in a series of 200 specimens of sputum that were negative on stained preparations of concentrated material. There were 36 instances in which a positive result was obtained by either one method or the other. The guinea pig method was responsible for 33 positive results and the culture method for only 3. The medium used was that of Sweany and Evanoff. There were no statistics on guinea pig mortality or contamination of cultures. It is not clear how many tubes were inoculated or the relative amounts of material apportioned to culture tubes and guinea pigs.

Feldman<sup>8</sup> found in the examination of clinical material that inoculation of animals is preferable because of its greater certainty. It is difficult to follow his line of reasoning because conclusions were based on a small series of 46 cases, 41 of which were positive by direct smear and only 5 negative. Of these 5, 2 were positive by animal alone and 3 were positive by both animal and

---

\*From the New York Hospital and Department of Medicine, Cornell University Medical College, New York.

Received for publication, January 29, 1938.

culture In the remaining 41 cases, as expected, comparable results were obtained by both methods Three different media totaling 18 tubes were used in each test

Later the same author, in collaboration with Magath,<sup>9</sup> reported on the examination of 100 specimens negative by direct smear The methods of Cooper, Swann, and Muirhead were tested Two thirds of the sediment was injected into guinea pigs and the remainder was seeded onto at least 8 tubes of media Contaminations ran from 5 per cent to 90 per cent, depending on the digestant and the media used There were 14 cases in which the animal alone was positive and only 4 cases where the culture yielded tubercle bacilli Failure to recover the tubercle bacilli in ten instances may have been due to the contaminated cultures

On the other hand, Norton, Thomas, and Broom,<sup>10</sup> using the Petriagani medium, concluded that the culture was the more sensitive method From 151 cases negative by smear, 54 positives were obtained, 35 by both guinea pig and culture, 16 by culture alone, and 3 by guinea pig alone Hirschberger<sup>11</sup> found cultures more sensitive in a series of 69 cases Cooper's, Hahn's and Muirhead's media were selected for this study and proved very satisfactory

From this brief review of pertinent literature one is left with the impression of a wide diversity of opinion concerning the efficacy of cultural methods It is also apparent that unless every step is scrupulously supervised the end results of such an investigation are apt to be fruitless or at least indecisive For that reason, it would not be unpardonable to criticize the soundness of some of the methods of study and the technique employed therein

There has been a fairly general tendency to inoculate the guinea pig with greater quantities of the suspected material than the culture tubes Frequently two to three different media involving 8 to 16 culture tubes were tested simultaneously, thus allowing a mere fraction of the total amount for each tube What is more important, where the cultures alone proved positive, few individuals determined the pathogenicity of these acid fast organisms by animal test

#### THE PRESENT STUDY

It was the purpose of the authors to select one suitable medium and submit it to a prolonged, rigidly controlled, parallel study with guinea pig inoculations In previous culture work in this laboratory, the Petriagani<sup>5</sup> medium had proved very satisfactory and therefore was our logical choice

Specimens were collected from patients in the Out Patient Department and medical pavilions of the New York Hospital All were from proved or suspected cases of pulmonary tuberculosis A total of 200 specimens comprising 137 sputa, 38 gastric washings, 21 pleural fluids, and a miscellaneous group of 4 was studied according to the procedure outlined below Smears made from the concentrated material of 171 specimens revealed no acid fast organisms Fifteen were slightly positive on smear and 14 markedly so These latter 14 were used at the beginning of this study to test out the method on known positives and as a further check on the efficacy of the medium chosen

# LABORATORY METHODS

---

## CULTURE VERSUS GUINEA PIG INOCULATION IN THE DIAGNOSIS OF TUBERCULOSIS\*

---

WILLIS A. MURPHY, M.D., AND DOROTHY RHOADES DUERSCHNER, A.B.,  
NEW YORK, N. Y.

---

IT WAS not until Dorset's description of his egg medium in 1902 that attention was focused upon the potentialities of cultural methods for the successful isolation of tubercle bacilli from contaminated material. It is beyond the scope of this paper to discuss all the media and their various modifications that have been brought forth since that time. Suffice it to say that the techniques of Petroff,<sup>1</sup> Corper,<sup>2</sup> Sweany and Evanoff,<sup>3</sup> Lowenstein,<sup>4</sup> and Petragnani,<sup>5</sup> among others have proved satisfactory in the hands of some investigators and unsatisfactory in others.

Herman, Hansman and De Capito,<sup>6</sup> using Corper's medium, studied a large series of 537 cases with both guinea pig inoculations and cultural methods. Of these, 48 proved positive by both methods. In an additional 28 cases the guinea pig alone became positive, whereas in 7 instances the culture alone became positive. Naturally, they concluded that animal inoculation was a more reliable procedure. Nevertheless, upon analyzing their cases, one notes that guinea pig fatalities accounted for twice as many failures as culture tube contaminations, drying of media, and breakage. The authors did not state whether they divided the material equally between the animal and the culture tubes.

In 1931 Stadnichenko and Sweany<sup>7</sup> compared the results of both methods in a series of 200 specimens of sputum that were negative on stained preparations of concentrated material. There were 36 instances in which a positive result was obtained by either one method or the other. The guinea pig method was responsible for 33 positive results and the culture method for only 3. The medium used was that of Sweany and Evanoff. There were no statistics on guinea pig mortality or contamination of cultures. It is not clear how many tubes were inoculated or the relative amounts of material apportioned to culture tubes and guinea pigs.

Feldman<sup>8</sup> found in the examination of clinical material that inoculation of animals is preferable because of its greater certainty. It is difficult to follow his line of reasoning because conclusions were based on a small series of 46 cases, 41 of which were positive by direct smear and only 5 negative. Of these 5, 2 were positive by animal alone and 3 were positive by both animal and

---

\*From the New York Hospital and Department of Medicine, Cornell University Medical College, New York.

Received for publication, January 29, 1938.

end of the fourth week. If macroscopic growth had not appeared by this time, the three tubes were examined in the same order at the end of the fifth, sixth, and seventh weeks. The cultures were discarded at the end of the eighth week. The date of the first appearance of growth, both microscopic and macroscopic, was recorded, likewise the presence and nature of contaminants.

*Examination of the Guinea Pig*—At the end of the second week after inoculation a tuberculin test was done on each guinea pig, using 0.1 cc. of a 5 per cent dilution of old tuberculin intradermally. This was repeated weekly until a two plus Mantoux appeared, or continued through the eighth week if the test remained negative. In any case the animal was sacrificed two days after the final test. An autopsy was performed on each pig and the presence of tubercle bacilli noted in the tissues before a positive interpretation was accepted as final.

### RESULTS

As will be noted in Table I, of the 171 specimens negative on stained preparations, 136 failed to become positive by culture or to produce tuberculosis in the guinea pig, 22 became positive by both culture and guinea pig inoculation, 9 by culture only, and 4 by animal test only. In most cases growth was

TABLE I

COMPARISON OF RESULTS OF CULTURES WITH GUINEA PIG INOCULATIONS ON CONCENTRATED SPECIMENS NEGATIVE BY SMEAR

NO. OF SPECIMENS	POS. BY BOTH GUINEA PIG AND CULTURE	POS. BY BOTH GUINEA PIG AND CULTURE	POS. BY CULTURE ONLY	POS. BY GUINEA PIG ONLY
119 Sputa	95	14	7	3
17 Pleural fluids	11	5	0	1
35 Gastric contents	10		2	0
Total 171	136	22	9	4

noted on all three tubes of each set. In each of the nine instances when the culture alone yielded acid fast bacilli, a portion of the visible growth was inoculated into normal guinea pigs. All of these animals became infected, gave positive Mantoux tests, and showed extensive tuberculous lesions at autopsy.

An additional 15 specimens, slightly positive on stained preparations, were also tested. Thirteen gave positive results by culture and animal inoculation. Two failed to become positive by either method.

Where the specimens were negative by smear, an average of thirty days was required for the culture to produce macroscopic growth, and an average of thirty three days for the guinea pigs to show positive Mantoux tests. Where the specimens were slightly positive by smear, the time required for the cultures and animals to become positive was approximately one half as long as when the smears were negative. As a rule microscopic growth was discovered about one week before characteristic colonies were visible.

As will be seen in Table II the percentage of guinea pig fatalities was considerably greater than the percentage of complete culture set contaminations. However the actual number of individual culture tubes contaminated was 25 out of a total of 558 tubes, or 4.5 per cent.

TABLE II

AN ANALYSIS OF UNCOMPLETED\* TESTS DUE TO GUINEA PIG FATALITIES AND CULTURE CONTAMINATIONS

NO. OF SPECIMENS TESTED	UNCOMPLETED TESTS*		PERCENTAGE UNCOMPLETED TESTS	
	GUINEA PIGS	CULTURE SETS	GUINEA PIGS	CULTURE SETS
186	11	2	6	1

\*Uncompleted tests refer to procedures which had to be abandoned because of the death of the animals or the contamination of all three tubes of a culture set before the time limit of eight weeks.

The contaminants encountered were of two kinds: those forming discreet colonies, and those forming spreading colonies. The latter gave the most difficulty because once they appeared, the entire surface of the slant was quickly overrun. The former, however, in only a few instances, rendered the culture tube worthless for the identification of colonies of acid-fast bacilli.

## COMMENT

In the early part of this article we have referred to the uncertainties that exist in the minds of investigators concerning the relative value of cultural methods and guinea pig inoculations. We have also placed great stress on the necessity of carefully controlling each step in any procedure used to determine the superiority of one method over the other.

Altogether there were 171 specimens negative on examination of the stained preparation. Of these, 31 became positive by culture as compared with 26 by guinea pig inoculation. Such an outcome indicates that the culture medium possesses a slightly greater sensitivity.

We have little comment to offer concerning the two occasions in which the cultures did not yield growth when the slides were positive. The corresponding guinea pigs succumbed to intercurrent infection in too short a period for development of tuberculous lesions. Although the culture method must be considered a failure in these two cases, it should be remembered that occasionally guinea pigs fail to contract tuberculosis after injection with faintly positive specimens.

The low incidence of contaminations in this series is noteworthy (4.5 per cent of the total number of tubes). We are convinced that this outcome could be attributed in a large degree to the fine state of dispersion of the material introduced upon the slants. Intimate contact of all the particles with the bacteriostatic agent was thereby assured.

Culture methods have certain very obvious economic advantages. In many communities, laboratories find it impossible to obtain guinea pigs. Where such a situation exists, cultures are recommended as a valuable supplement to routine slide examination.

In laboratories where guinea pigs are readily available and in routine use the culture technique affords additional diagnostic aid.

## CONCLUSION

Petragnani's medium is an effective agent in the isolation of tubercle bacilli from contaminated material. In our hands, the cultural method employing this medium appears to possess a slight superiority over the guinea pig inoculation.

## REFERENCES

- 1 Petroff, S A Variations of the Tubercle Bacillus When Cultivated on Potato Media, Nat Assn Study & Prev Tuber Tr 9 341, 1913
- 2 Corper, H J, and Uyei, N Further Observations With a New Method for Cultivating Tubercle Bacilli A Comparison With Guinea Pig Inoculation and Petroff's Method, J LAB & CLIN MED 14 393, 1929
- 3 Sverny, H, and Linnoff, M Further Studies on the Cultivation of the Tubercle Bacillus Am Rev Tuberc 18 661, 1928
- 4 Lowenstein, E La methode d'hémoculture du virus tuberculeux et ses resultats, Ann Inst Pasteur 50 161, 1933
- 5 Petragnam G Medium Am Pub Health Assn Year Bool 1935 36, p 191
- 6 Herman, W W, Hansman, G H, and De Capito, T The Demonstration of Myobacterium Tuberculosis in Exudates, Tissues, and Body Fluids Concerning Guinea Pig Inoculation and Cultural Method for the Demonstration of Myobacterium Tuberculosis, Am J Clin Path 1 371, 1932
- 7 Stadmichenko, Asya, and Sverny, H C A Comparison of Culture and Animal Inoculation of Sputum in the Diagnosis of Tuberculosis, Am J Clin Path 1 303, 1931
- 8 Feldman, William H A Comparison of the Different Culture Methods for the Isolation and Growth of Myobacterium Tuberculosis, Am J Clin Path 1 285, 1931
- 9 Magath Thomas B, and Feldman, William H The Relative Value of Cultural Methods and Guinea Pig Inoculation in the Diagnosis of Tuberculosis, Am J Clin Path 2 199, 1932
- 10 Norton, John F, Thomas, James G, and Broom, Norma H Laboratory Tests for Tubercle Bacilli by Culture Methods, Am Rev Tuberc 25 378 1932
- 11 Hirschberger, Nell An Evaluation of Three Methods for the Demonstration of Tubercle Bacilli for Use in Hospital Routine, J LAB & CLIN MED 19 429 1933

## MODIFIED VAN ALLEN HEMATOCRIT TUBE PROVIDING FOR AUTOMATIC VOLUME ADJUSTMENT OF THE BLOOD SAMPLE\*

GEORGE M GUEST, M D, CINCINNATI, OHIO

THE pipette here described was designed for the determination of the volume of cells in blood according to the Van Allen (1925) method, and to facilitate measurements of cell volume in a method for the determination of red blood cell fragility which will be reported later. It offers advantages over the original Van Allen hematocrit tube by providing quicker and more precise measurement of the blood sample this being accomplished by automatic volume adjustment of the sample in a capillary tube similar to that used in the Tienner diluting pipettes employed for counting blood cells.

The Van Allen hematocrit method possesses the advantage of requiring only a small amount of blood, obtainable by skin puncture, furthermore, in this method packing of the cells to constant volume can be attained in a relatively short time and at a relatively low centrifuge speed because the blood is diluted in a salt solution having lower specific gravity and viscosity than the blood plasma. Against these advantages may be cited the objections that the rubber sealing device may permit leakage during centrifugation, and that the diluting fluid, although apparently isotonic for normal blood, may not be isotonic for bloods from subjects with various pathologic conditions. Such considerations as these must be weighed carefully when a choice is made of any one of the many hematocrit methods which have been devised for cell volume measurements.

\*From the Children's Hospital Research Foundation and the Department of Pediatrics University of Cincinnati

In the procedure as originally described by its author, blood is drawn into the Van Allen hematocrit tube up to the 100 mark, excess blood is wiped from the tip, and a diluting fluid (1.3 per cent sodium oxalate solution) is drawn into the tube until the bulb is about half filled. The tube is sealed—either with a rubber band or with a spring clip device which holds a rubber cushion against

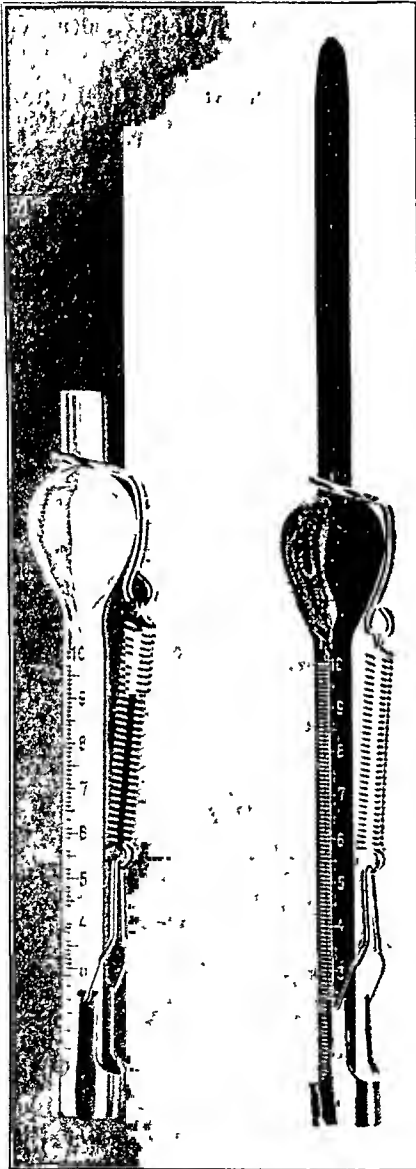


Fig. 1.

Fig. 1.—Original and modified Van Allen hematocrit tubes.

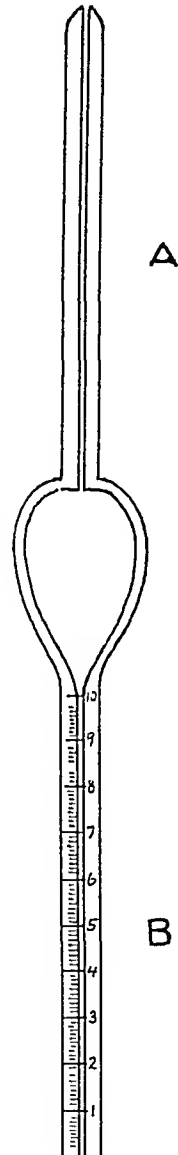


Fig. 2.

Fig. 2.—Diagram showing construction of the modified tube.

the tip—and is then centrifuged. The height of the column of packed cells, read from the graduations on the stem, gives the percentage volume of cells in the sample.

In practice, accurate adjustment of the blood sample in the original tube is somewhat tedious and subject to error, inasmuch as the broad flat tip makes

difficult the wiping of excess blood from the end of the tube without pulling the column of blood away from the 100 mark. The modified tube shown in Fig 1 was designed primarily to overcome this difficulty. For such a modification it was, of course, not possible merely to substitute the Tiemmer type of capillary tube for the graduated stem of the original tube, because the blood cells when centrifuged would then settle at the flat shoulder inside the bulb instead of in the graduated stem where the column of cells is measured. Both ends of the pipette, therefore, were utilized, one for measurement of the blood sample, the other for measurement of the column of packed cells after centrifugation.

*Construction*—The capillary tube A is tapered at the tip, to facilitate wiping away of excess blood, and at the opposite end it terminates in a ground and polished surface at right angles to the longitudinal axis. The volume of the bulb has been made (arbitrarily) approximately 100 times that of tube A. It is formed separately, blown from tube B in order to provide a smooth funnel shaped opening into the latter, and is fused to the shoulders of tube A. The graduated portion of tube B has a volume exactly equal to that of tube A, and is marked with 100 divisions numbered by tens from the distal end.

In use, a rubber suction tube with mouthpiece is first fitted to the end of tube B. With the pipette held horizontally or with the B end slanted downward blood is drawn into tube A by gentle suction until it will flow by capillary to fill the tube A completely. After excess blood is wiped from the tip, diluting fluid is drawn into the bulb by suction until the bulb is nearly filled. An air bubble left in the bulb aids the mixing of the blood with the diluting fluid. The suction tube is then removed, the stem B is sealed with a spring clip (the same as that used with the original Van Allen tube) which holds a rubber cushion against the blunt end, and the tube is centrifuged with the sealed end downward. The cells are thus packed into the graduated stem B, and there measured directly in percentage of the volume of the sample by reading of the graduations.

Since a rather thin edge of the bulb is fused to the outside of the shoulder of tube A, this modified tube is necessarily more fragile than the original form, and there is greater danger of breakage at high centrifuge speeds. In order to avoid torsion stress, the tube should be placed in a centrifuge cup which holds it in an exactly perpendicular position. The International Centrifuge, type SB, No 1 or 2, may be used with the 15 ml centrifuge tube carriers. The rubber cushions usually placed at the bottom of the metal cups are not needed. These cups can be filled with water above the shoulder of the bulb to give additional support in counterbalancing the centrifugal force against the bulb.

#### SUMMARY

The Van Allen hematocrit tube has been modified to provide quicker and more precise measurement of the blood sample, by utilizing the principle of automatic volume adjustment of the sample in a capillary tube similar to that used in Tiemmer hematometer pipettes.

This pipette and spring clip sealing device is obtainable from the Arthur H. Thomas Co. Philadelphia.

#### REFERENCES

- Van Allen, C. M. An Hematocrit Method, *J. LAB. & CLIN. MED.* 10: 1027, 1925.  
 Van Allen, C. M. Sealing Device for Hematocrit, *J. A. M. A.* 85: 2033, 1925.



## AN IMPROVED MANTEL FOR BACTERIA-PROOF FILTER CANDLES\*

R. R. HENLEY, M.S., WASHINGTON, D. C.

THE glass mantels commonly used with bacteria-proof filter candles have several inherent disadvantages: they are breakable, the liquid undergoing filtration is exposed continuously to air-borne contaminants, and repeated re-fillings requiring frequent attention are necessary when large volumes are filtered. A metal mantel free from these disadvantages, and particularly serviceable for the filtration of large volumes of products is shown in Figs. 1 and 2.

The mantel of which the dimensions are given in Fig. 1 will accommodate a 5 inch filter candle and a 4 liter filter flask. The metal used has been duralumin, which is obtainable in bars and tubing of the requisite sizes. The hole through the base of the mantel is threaded to fit the nipple of the candle. If a suitable tap is not available, one of the metal nuts always supplied with candles may be sweated into a hole countersunk in the "boss" of the base. The glass tube and rubber stopper shown may be replaced by a metal cap threaded to fit the top of the cylinder and provided with a suitable nipple and washer. The top of the flask upon which the mantel rests must be ground flat if necessary.

Three different types of rubber washers are used. The washer between the candle and the boss is cut from  $\frac{1}{4}$  inch sheet rubber packing provided with cloth insertion. It is used once and then discarded. The washer between the cylinder and the "boss" is a standard 2 inch hose washer. The large washer between the "boss" and the flask is cut from  $\frac{5}{16}$  inch grade A rubber sheeting. The latter two washers are used as long as they remain serviceable.

The set-up of the apparatus is shown in Fig. 2. Prior to sterilization, the mantel, candle, and suction flask are assembled, and the space between the inner wall of the base of the mantel and the outer wall of the flask is packed with cotton. The assembled apparatus with supply tube attached or not, as preferred, is then sterilized.

In operation one end of the supply tube with the clamp closed is attached to the inlet tube of the mantel, and the other end is immersed in the liquid to be filtered, which should be contained in a reservoir plugged with cotton. The system is thus protected from air-borne contaminants. The suction flask is connected to the vacuum line, and suction is applied with simultaneous downward pressure upon the mantel. When the collapse of the supply tube indicates exhaustion, the clamp is opened, the mantel fills, and the filtration proceeds automatically. With suitable precautions for the prevention of contamination,

\*From the Biochemic Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

Received for publication, February 16, 1938.

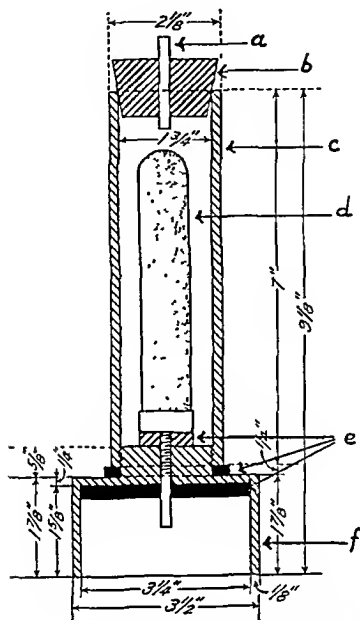


Fig 1—*a*, Glass inlet tube, *b*, rubber stopper, *c*, metal cylinder, *d*, candle, *e*, rubber washers, *f*, metal base

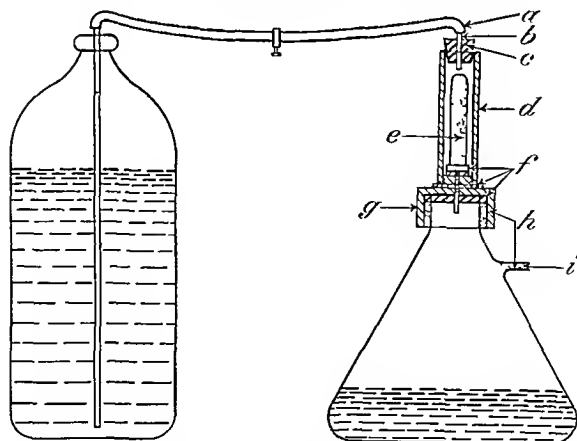


Fig 2—*a*, Rubber supply tube, *b*, glass inlet tube, *c*, rubber stopper, *d*, metal cylinder, *e*, candle, *f*, rubber washers, *g*, metal base, *h*, cotton, *i*, to vacuum line

the mantle may be transferred from one flask to another. Since the only attention required is the turning on and off of the suction, a number of filters may be connected to a single reservoir and operated simultaneously.

The apparatus has been used chiefly for the filtration of intradermic tuberculin which contains 0.5 per cent phenol. During the past several years more than five million cubic centimeters have been filtered and the filtrates examined by cultural and microscopic methods. No contamination has been traced to a fault or defect in the mantle.

---

## A NEW STOMACH TUBE FOR ORAL ADMINISTRATION IN RATS AND MICE\*

---

A. E. PUGH, M.S., AND A. W. TANDY, JR., B.S., KANSAS CITY, MO.

---

**I**N A SERIES of experiments on mercury intoxication, it became necessary to give rats and mice oral administrations of small, accurately measured doses of liquids. The customary method, using a small rubber catheter, was found to be neither accurate nor rapid enough for this work.

An instrument satisfactory for this study was made by modifying an 18 gauge hypodermic needle of length sufficient to pass into the stomach of the animal being used. The beveled point of the needle was removed on an emery wheel and the last 2 cm. of the end of the needle was covered with a layer of solder several millimeters thick. This end was then shaped on a lathe until a smooth blunt tip, about 2 mm. in diameter, with a gradual taper to the shank of the needle remained. For adult rats, a needle 4 inches in length allows insertion into the stomach. For mice a 20 gauge needle, 2 inches long with a ball tip of about 1 mm. thickness, should be used. The needle is attached to a syringe of capacity sufficient to hold the dose being administered.

### TECHNIQUE

At first it is best to have two operators, although after a little practice one person can perform the operation. One operator holds the head and extends the tongue of the animal while the second inserts the needle into the stomach, manages the syringe, and holds the animal's tail.

The first operator takes a full grasp of the skin on the back of the animal, extending from immediately behind the ears as far down the back as a grip between the thumb and the full medial side of the index finger of the left hand allows. The middle or third finger of the left hand pins the left front leg against the animal's body. The thumb does not go around the throat but the full weight of the animal is maintained by the scruff of the neck. With the right hand, the tongue is grasped firmly with blunt dressing forceps and drawn out to the left side of the animal. This opens the mouth and allows the insertion of the needle deep into the pharynx. The tongue is released after the needle has passed over

---

\*From the Research Laboratories of the George A. Breon & Co., Inc.  
Received for publication, February 19, 1933.

the papilla at its base and the right hand may then be used to grasp the left cheek of the animal to draw the head and neck in line with the body if the animal insists on arching its neck.

The second operator holds the body of the animal straight by maintaining a steady pull on the animal's tail with the left hand. With the right hand he inserts the needle and operates the syringe. The index finger of the right hand should be used to bind the plunger of the syringe against the syringe wall to prevent leakage of the solution through the needle while inserting. The needle should be passed into the mouth in line with the animal's body and just to the left of the incisors with the blunt end against the palate. By a gentle lever action on the syringe forcing the head back as the end of the needle approaches the larynx, the needle passes easily over the glottis into the esophagus and enters the stomach without difficulty.



Fig. 1.

Fig. 2.

Fig. 3.

Fig. 1.—Stomach tube hypodermic needle with enlarged blunt tip

Fig. 2.—Introducing tube into mouth of rat. Note extension of tongue

Fig. 3.—Passing tube into stomach. Note straightening of neck by pull on cheek

When the injection is made by one person, the left hand holds the animal by the scruff of the neck and the right hand inserts the needle by feeling the way over the tongue and using a gentle leverage with the syringe to force back the animal's head.

Two injections per minute can be accomplished easily with doses under 5 c.c. With larger doses time must be allowed for the stomach to relax, otherwise the esophagus will be filled and part of the injection lost through the mouth and nose. Since the perfection of this apparatus over 7,000 injections (as high as 300 injections on one rat) have been made in this laboratory without the loss of a single animal from trauma or drowning.

The photographs illustrate the stomach tube and the various steps in its application on the rat.

# THE GEIGER-MÜLLER COUNTER\*

## A RECENT INSTRUMENT IN MEDICINE AND MEDICAL RESEARCH

M. L. WEINSTEIN, M.D., AND LEOPOLD ROVNER, M.S., CHICAGO, ILL.

A nontechnical description of a physical apparatus that has been finding applications in the field of medicine, especially in radiology, in radiobiology, and in physiology. It is the purpose of this article to describe the properties of the apparatus sufficiently well so that its function and some of its possibilities and limitations may be a matter of general record. A brief bibliography is included with references to significant historical and technical articles in the literature.

### I. PROPERTIES AND USES OF THE GEIGER-MÜLLER COUNTER

WITHIN the past few years an instrument that had been primarily designed for physical studies of the atom (and more recently, for research in cosmic rays) has emerged from the physics laboratory to become a useful part of the general armamentarium of medicine. The instrument, known as the Geiger-Müller counter, is a physical apparatus for observing ionizing radiations of very low intensity.

The counter has an extreme sensitivity for this purpose since it is able to record the entry of a *single electron*, or the absorption of a *single photon* (light corpuscle) within the limits of the sensitive chamber of the apparatus.

It is interesting to observe this unique property of the apparatus in comparison with even the most sensitive radiation detectors of more familiar usage (in various regions of the spectrum): thermopiles, photocells, air-ionization instruments, each of these being an apparatus which generally requires the incidence of many thousands of photons per unit area per second to produce an appreciable effect for observational purposes.

The counter is finding certain practical applications in medicine not only as a consequence of this property of extreme sensitivity, but also because of the fact that observations made with the instrument on ion-producing radiations of low intensity (and thus, for example, on very minute quantities of radioactive material) can be quite accurately made, and are consequently capable of quantitative interpretation.

In radiology the instrument has been used for detecting harmful stray radiations from x-ray and radium equipment. It is now the most practical device for finding lost radium,<sup>§</sup> and has already figured in many successful searches.† (Newspaper accounts of these interesting radium hunts refer to the apparatus as the "electric detective" or the "radium hen".)

\*From the Prothero Radium Memorial, Chicago Memorial Hospital.  
Received for publication, February 28, 1938.

†Dr. R. B. Taft, of Charleston, S. C., has prepared an interesting history of these stories of lost and found radium in book form: *Radium Lost and Found*, Charleston, S. C., 1938, J. J. Furlong & Son.

It is, however, in radiobiology and in physiology that the instrument may make contributions that are especially significant to medicine. During the past five years various means have been discovered for producing a temporary radio activity in the atoms of many of the chemical elements (without changing their ordinary chemical behavior). These radioactivated elements and the Geiger counter have together opened a new avenue for tracing foodstuffs in the metabolic process,<sup>1</sup> or for observing the transfer of chemical substances during various physiologic activities.

The instrument has recently proved of great value in the quantitative physical detection of slight traces of radium in living victims of radium poisoning.<sup>2</sup> Although the total quantity of radium distributed throughout the body of an adult individual may be as little as two or three thousandths of a milligram, this amount can be determined with accuracy and with considerable speed by means of the Geiger counter.

## II THE INSTRUMENT IN RADIOLOGY EXAMINATIONS OF THE SKIN

An important problem to be met in x-ray and radium therapy institutions is that of protecting the personnel from harmful stray radiations. Several national radiotherapy and radiologic physics centers have provisionally accepted as "safe" those working conditions in which the intensity of the stray penetrating radiation is about 0.2 roentgens in eight hours or  $4.2 \times 10^{-4}$  (0.00042) roentgens per minute.<sup>3</sup> (For comparison, a total of 600 roentgens of filtered 200 kv x-rays is necessary to produce a perceptible erythema on the human skin.)

We have built some Geiger Muller instruments (Figs. 1 and 2) that have, among other things, served us in qualitative observations of stray radiation and radiation 'leaks'. The counter tubes of our apparatus have elements of such a size that, under test, each of the instruments was found to double its 'natural count', of 10 impulses per minute with a stray radiation intensity of about  $1.7 \times 10^{-4}$  (or 0.0000017) roentgens per minute.

This great apparent sensitivity is a real one in terms of roentgens. For clarity, however, it should be noted that the 'roentgen' is a unit quantity of x-rays or of gamma rays corresponding to an enormous number of photons. The individual unit particles of radiation in a directed beam of gamma rays having an intensity of one roentgen per minute at a given point, sends about  $2 \times 10^9$  or 2,000,000,000 gamma ray photons per minute perpendicularly through a one square centimeter area at that point.

The arbitrarily safe intensity of  $4.2 \times 10^{-4}$  roentgens per minute would ideally cause our own apparatus to respond with 25,000 impulses per minute. Such an intensity would be physically too great for our own rather simple apparatus to record quantitatively; paradoxically, we are able to make quantitative measurements only on radiations having  $\frac{1}{2}$  to  $\frac{1}{25,000}$ th of the magnitude of this small intensity. (As shall be briefly explained later, Geiger Muller counter instruments are usually built to suit certain ranges of radiation intensity, the tube element used being generally made smaller for greater intensities. There are circuits that permit counting up to 200,000 impulses per minute.)

It should be noted by those using similar types of Geiger Muller apparatus to observe the presence of stray radiations, that a large number of impulses may be registered under safe radiation conditions. The high sensitivity of such apparatuses may make it seem that

<sup>1</sup>This figure may be materially reduced because of recent considerations of the effects of even such low intensities of radiation in producing mutations in the germ plasma.

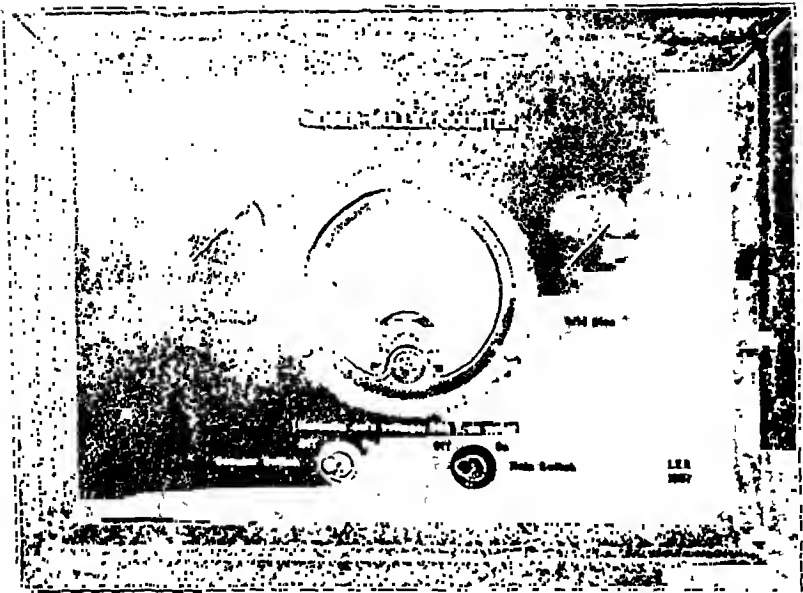


Fig. 1.—Counter built for operation on 110 volt AC line.

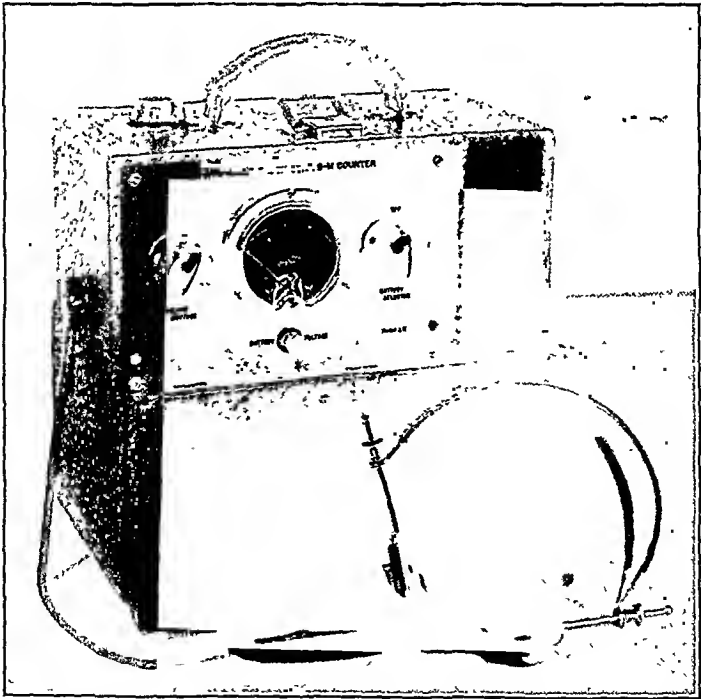


Fig. 2.—Battery operated counter instrument, independent of all external lines.

an alarming amount of radiation is present in a room, whereas (at an observed rate increase of 30 to 50 counts per minute) there may be only a few thousand photons passing through the counter tube each minute—a flux representing a truly safe amount of radiation in the room, as may easily be recognized by expressing this flow of photons in terms of fractional roentgens.

Lost radium is quickly traced with Geiger-Müller instruments, since the gamma radiation given off by the radium can be detected at an appreciable distance. Using the relatively small size element that is found convenient for our own apparatus, we get twice the natural count of about 10 impulses per



Fig. 3—Battery operated instrument in use.

minute (due to intrinsic radioactivity of the surroundings and to cosmic rays), that is, twenty impulses per minute, with one milligram of radium at thirty feet free air-line distance.

Fig. 3 shows how the instrument is carried in a radium search (or in a stray radiation survey). We use the Geiger-Müller tube mounted within the case for mechanical and electrical protection. As viewed in Fig. 3, the tube\* is mounted in the lower right hand corner of the case with its axis parallel to the lower leading edge.

\*The tube, itself, and its properties are described in a following section.



## III. G-M COUNTERS IN TOXICOLOGY: RADIUM POISONING, STUDIES OF LEAD POISONING

In biological investigations the counter apparatus is most generally used as a delicate assay instrument. With it one may measure quantities of certain radioactive substances that are present in amounts ordinarily determined only by microchemical methods. There are, indeed, instances where a microchemical method cannot be used, or is less sensitive than the measurement with the counter instrument.

One such example occurs in the diagnosis of radium poisoning. The quantity of radium element producing lethal effects in the human being is of the order of magnitude of one microgram. This small amount of radium may be distributed at random throughout the whole skeleton of a living individual. Under these difficult conditions it is nevertheless possible to detect chronic radium poisoning at any time by a physical procedure, whereas an interval of five to ten years can elapse before there may be an appearance of clinical symptoms. The detection, a quantitative one, is made by a relatively simple counter examination of the patient, through observation of the very weak gamma radiations emitted from his body.

Electroscopes, previously used for such work, are less sensitive than the counter, sometimes failing to detect, even qualitatively, the presence of an ultimately lethal amount of radium.

R. D. Evans<sup>2, 3</sup> and his colleagues have been chiefly responsible for developing a complete method: suitable counter and other important observation instruments, and an adequate technique, for the recent excellent quantitative physical studies made on radium poisoning. Evans<sup>2</sup> has devised a general method of determining the amount of a gamma ray emitting substance in a closed and inaccessible container. His method takes account of a random distribution of the radium in various bones and tissues, and of the internal absorption and scattering undergone by the gamma rays, so that a correct evaluation of gamma ray emitting substance within the body (radium C) may be made after a series of observations on the individual living person.

The complete physical study of radium poisoning of the individual involves other apparatus: gas collectors, sensitive ionization-electrometer instruments to record the amount of gamma radioactive principle (radium C) escaping in the exhaled breath as radon (a gaseous disintegration product of radium), and to observe the amount of radium being eliminated in the feces and urine.

(The total radium in the body is a quantity represented by the sum of the amount of radium C observed in the breath and of the radium C in the body itself. Radium, alone, is not appreciably gamma radioactive, it is the relatively short-life disintegration product radium C which emits most of the useful gamma rays.)

Using measurements made on several individuals, the data of which show a representative distribution of the radium in the skeleton, Evans has directly calibrated his counters for rapid measurement of other persons, and can make a fairly accurate survey of a single patient in from fifteen minutes to one hour with just the counter instrument.

Some of the results of this study prove quite interesting observations show that 45 per cent of the radon produced by the radium in the body is exhaled with the breath, that cases of chronic radium poisoning normally eliminate 0.005 per cent of total body radium per day (as shown by earlier investigations), and that this small outgoing quantity distributes itself with considerable constancy in a percentage ratio of 91 per cent in the feces to 9 per cent in the urine. The work suggests the use of small traces of the radioactive lead isotope, radium D, as an indicator in a lead mixture for lead poisoning studies.

It is interesting to note that in 1923 Hevesy\* had already used a radioactive lead isotope (thorium B) to trace the absorption and translocation of lead in plants as a measure of lead metabolism, and also as a measure of the toxicity of lead. Hevesy ignited various portions of the plant and determined the active lead content of the ash concentrate with an electroscope. By adding radioactive isotope (to the inactive lead) in amounts that were increased in direct proportion to the extent of total dilution of lead in the nutrient medium, he was easily able to make measurements on very weak dilutions of lead, and thereafter to observe the disposition of a few hundredths or few thousandths of one per cent of the amount of lead initially made available to the plant.

Reeves and Morgan† following a preliminary report by Taft,‡ have used the counter instrument to measure the retention of thorium dioxide (Thorotrust) within the human subject, after injection of the material for x-ray diagnostic purposes. (The weak gamma ray emission of this material serves as an indicator of its presence, while the amount of radiation measured gives an approximation to the actual content of thorium dioxide within the subject.) Reeves and Morgan make no statement on the toxicity of the substance, but conclusively demonstrate its retention in large amounts even four years after the initial injection.

#### IV. USE OF THE G-M APPARATUS IN PHYSIOLOGY

From the statements of the foregoing section, it becomes apparent that the counter instrument forms one part of a dual team—of radioactive substance and sensitive detector—for investigating the fate of “tagged atoms” in physiologic processes. The English physiologist, A. V. Hill, has stated that artificially radioactive substances may have a meaning to present day biology much like that which the microscope had for an earlier era. Then the microscope revealed the cell, today, some aspects of the physiologic fate of the atoms themselves may be visualized with the new observational technique.

There are many examples available of the uses to which activated substances and counter instruments have already been put in physiologic investigations. The general procedure has been to “tag” the atom of a particular element within a compound, for example P, ordinary phosphorus (which is  ${}^1_1\text{P}^{31}$ ) in  $\text{Na}_3\text{PO}_4$  is identified by intermixing with that compound an identical combination of a radioactive isotope of the element (Example  ${}^1_1\text{P}^{32}$  in the  $\text{Na}_3({}^1_1\text{P}^{32})\text{O}_4$  molecule.)

\*Hevesy G. *Biochem J* 17: 439 1923

†Reeves R. J. and Morgan J. D. *The Retention of Thorium Dioxide by the Reticulo-Endothelial System* *Radiology* 29: 612 1937

‡Taft R. B. *The Radio Activity of Thorium Dioxide Solution* *J. A. M. A.* 108: 1779 1937

§15 is the atomic number and 31 is the atomic weight

Since the general chemical behavior of elements and their isotopes is identical in character, the labelled atom goes through the same molecular interchanges as its inactive brothers, and thus serves to reveal where they have gone. (It should be noted that this argument is true insofar as the radioactivity is so weak that no radiation changes are produced, for example in a colloidal system within which the radioactive substance may be.)

Simply considered, the basic process of making quantity observations with the counter is briefly this: the substance administered is measured under fixed conditions and rated at a given radioactivity factor, say, 1000. If, thereafter, some portion of the initially administered substance—suppose it be that contained within a complete thyroid gland—is found to have a radioactivity of 10, then one may say that 1 per cent of the material initially given found its way into the thyroid tissue.

Chiewitz and Hevesy<sup>1</sup> have made some studies on phosphorus metabolism in the rat, using radiophosphorus, with the counter apparatus as the assay instrument. The rats were fed a few milligrams of sodium phosphate containing  $^{32}\text{P}$ , and their separate organs and skeletal systems were later examined for radiophosphorus. These examinations showed that incoming phosphorus existed in certain characteristic ratios in the skeleton, in the spleen, kidneys, and brain. More particularly, in following the rate of elimination of  $^{32}\text{P}$ , the authors deduced that the average time which a phosphorus atom spends in the organism of a normally fed rat is about two months. They felt that their results lent more support to the view that the maintenance of bone structure is a dynamic process, the bone continually taking up phosphorus atoms, all or some of which are lost to be replaced again by incoming phosphorus. In examining different parts of the skeleton, Chiewitz and Hevesy found a conspicuous active phosphorus content in the teeth of the rat. The front teeth, which grow rapidly, contained a larger part of the incoming phosphorus, per gram, in a ratio of 10 to 1 (as compared with the whole skeleton) for an adult, and 6 to 1 for a young, growing animal. The molar teeth were found to take up less than the average amount of phosphorus per gram of basic weight, having a ratio as low as 1 to 2 compared with the skeleton.

In several investigations in plant physiology Hevesy and his associates\* have performed some interesting and suggestive experiments that may readily find counterparts, as experiments, in animal physiology; for example, germinating maize and pea seeds were found to take up the labelled phosphorus in the germ but not in the endosperm, showing that there is no phosphorus exchange between the two.

Hamilton<sup>7</sup> has done some work which is useful in suggesting a method for studying the rate at which various elements pass from the digestive tract into the blood stream.

The present form of the experiment is quite interesting, though it is not truly quantitative in terms of the physiologic process involved: a subject receives, by mouth, a water solution of sodium chloride containing radiosodium (a short-life isotope of sodium which disintegrates with the ultimate emission

\*Hevesy, G., Linderstrom-Lang, K., and Olsen, C.: *Nature* 137: 66, 1936; 139: 149, 1937.  
Hevesy, G., Linderstrom-Lang, K., and Nielsen, N.: *Nature* 140: 725, 1937.

of gamma radiation) The subject holds in his hand a Geiger Muller tube that is heavily shielded from the rest of his body by thick layers of lead As the active radiosodium diffuses into the blood stream and passes through the vessels of the hand, the counter responds with an increasing barrage of impulses A curve can be plotted showing a characteristic variation of the observed impulse rate with time, as increasing amounts of radiosodium reach the hand holding the counter tube The exact shape of the curve is modified by several physiologic factors—which may be subject to inspection themselves, because of the modifications they produce

#### V HISTORY OF THE COUNTER, GENERAL PRINCIPLES OF ITS ACTION

While the counter has become available in a stable and rugged form only within the past two or three years, the essential operating principle of the instrument is not new

In 1908 Rutherford and Geiger were making studies of the emission of alpha particles (positively charged helium nuclei) from the radium atom, and decided to use some device that would make a single one of these charged atomic particles detectable with the laboratory electrometer They invented the novel principle of having the single charged particle initiate a sudden flow or discharge of a relatively large quantity of electricity between two electrodes in a rarefied gas chamber This principle was based upon the known process of automatic increase of gaseous ionization by ionic molecular "collision"

Alpha particles from a radioactive source were allowed to pass through a very thin aluminum foil "window" into a brass tube (see schematic drawing in Fig 5A) containing gas at about one eighteenth of normal atmospheric pressure (The brass cylinder itself [the cathode] and an inner central wire [the anode] served as the two electrodes each being insulated from the other by ebonite plugs at the ends of the cylinder The voltage between cylinder and wire was adjusted to a critical value just below that which would ordinarily produce a flashover, approximately 1300 volts in this case)

As a single alpha particle sped past the window and then through the gas in the chamber, it left in its wake a large number of free ions split off from the neutral gas molecules These nearly stationary ions in the wake of the fast particle were immediately swept up by the field and sent careering toward the electrodes, crashing into neutral molecules on their way and forming new ions by the same process with which they themselves had initially been released by the speeding alpha particle The ionization was thus automatically and continuously increased until there were enough ions available to carry a surge of electricity through the circuit to the electrometer, which deflected electrostatically to announce the initial event—the entry of one charged atomic particle (The whole process of the growth of the discharge in this way easily took place in less than one ten thousandth of a second)

In 1928 Geiger and Muller made an important addition to the principle of action of the apparatus by utilizing the metal wall of the chamber, now enclosed in a glass envelope, (see Fig 5B) as the chief source of ionizing particles for the interelectrode gas volume In this particular case the discharge was initiated by single electrons, either those originating uniquely from among the atoms of the cylinder material\* or those that were released in and about the walls of the gas chamber by the absorption of photon radiation

\*As in the purposeful use of a cylinder made of pure potassium for the study of the very weak radioactivity of that metal



Photon or electromagnetic radiations (all of which travel with the speed of light, whatever their energy content) may enter a counter tube through the proper transparent aperture. Fig 4B is a representation of the sort of tube used for very weak visible and ultraviolet light. It is essentially a gas filled photoelectric cell and Geiger-Müller tube combined in one. A single photon may enter through the transparent plate, and, upon being absorbed by one atom of the cathode surface, causes the emission from there of a single photo

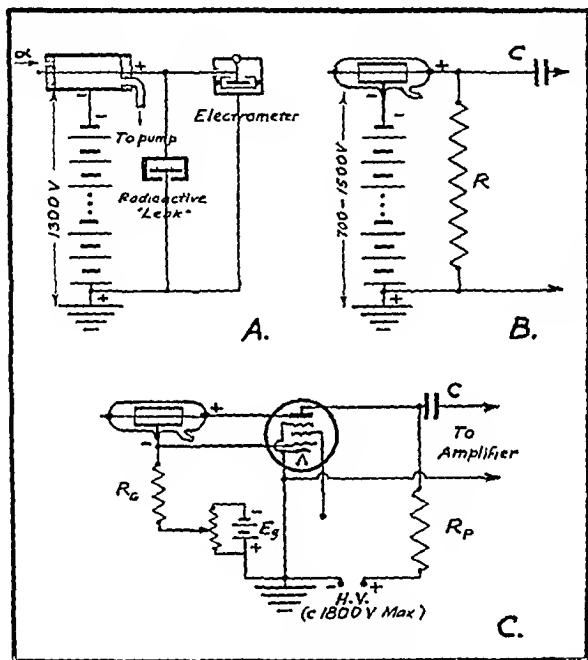


Fig 5—Types of counter circuits

electron—a charged particle that is thereupon put to work initiating the trigger mechanism that announces the unique event.

Since photons of the familiar x ray and gamma ray regions of the spectrum are quite penetrating, no special window or other aperture is necessary to admit the radiation to within the confines of the sensitive chamber of the tube. Fig 4C shows the type of counter tube used for penetrating radiations. The tube element is made of some metal, such as copper or silver, which has a satisfactory absorption coefficient and adequate wall thickness for the radiation in question.

The tube intercepts radiation directed perpendicularly toward its axis in proportion to the longitudinal cross section area of the metal cylinder element

For intensities of gamma radiation, such as those from 1 to 10 mg. of radium at 20 to 30 feet, one might use elements of 2 to 3 cm. length and about 1 to 2 cm. diameter. For cosmic ray particles (which reach sea level at the rate of about one particle per 10 cm. square of horizontal area per second) one may have counter tube elements of as much as 50 cm. length (or greater), and 2 to 10 cm. diameter.

Three counter circuits are known in Figs. 5A, B, and C. These circuits, especially the last two, are of the type essential for the operation of the counter tube itself, and will be briefly explained. For the complete measuring instrument it is necessary to have the proper high voltage supply and proper impulse amplification and impulse recording circuits; these are mentioned in the last section.

Fig. 5A shows the initial circuit used by Rutherford and Geiger; it is now of historical interest, but the parallel between it and the succeeding types is quite distinct.

The circuit of Fig. 5B delivers pulses to the amplifier in the following manner: the flight of one ionizing particle through the sensitive volume of the counter tube (or the liberation of a single electron within its confines) immediately initiates the process of collision ionization. Within a time interval of the order of one hundred thousandth of a second the full discharge is formed, and a current flows around the circuit of voltage source, counter, and resistor ( $R$ ). Passage of the minute current through the high ohmage resistor creates a gradient of voltage there (the  $IR$  drop) which polarizes the small condenser ( $C$ ) and which then very shortly reaches a value sufficient to cut the potential across the counter tube down below that at which a discharge can be maintained. The discharge in the counter tube is thus "extinguished" by the current flow through the resistor. Thereafter the potential difference between the electrodes of the counter tube is restored while the charge flows off the small condenser toward the ground. During this interval of about one ten-thousandth to one-tenth of a second (depending upon the amount of capacity and resistance in the circuit) a "wave" of electrical polarization has passed over the plates of the small condenser and is impressed upon the amplifier circuit, which ultimately actuates a loud speaker in a sharp "click," flashes a neon tube, or drives an electrical or mechanical recording mechanism.

The circuit of Fig. 5C uses a vacuum tube to extinguish the discharge.<sup>9</sup> After the initial ionizing event, the current starting through the counter tube and  $R_G$  polarizes the control grid,  $G$ ,\* which releases a relatively large surge of electricity through the vacuum tube and resistor  $R_P$ ; the current through the vacuum tube produces a large  $IR$  drop in  $R_P$ , and thus (in lowering the available voltage) effects rapid extinction of the discharge in the counter tube. Thereafter the charge on the coupling condenser ( $C$ ) is permitted to flow to ground in a short time interval through the relatively small extinguishing resistor,  $R_P$ . The circuit is consequently restored to its initial sensitive state in a very brief interval of time.

\* $G$  must be maintained at a critical negative value by means of the small battery  $E_g$  for proper operation of the circuit.

For intensities of gamma radiation, such as those from 1 to 10 mg. of radium at 20 to 30 feet, one might use elements of 2 to 3 cm. length and about 1 to 2 cm. diameter. For cosmic ray particles (which reach sea level at the rate of about one particle per 10 cm. square of horizontal area per second) one may have counter tube elements of as much as 50 cm. length (or greater), and 2 to 10 cm. diameter.

Three counter circuits are known in Figs. 5A, B, and C. These circuits, especially the last two, are of the type essential for the operation of the counter tube itself, and will be briefly explained. For the complete measuring instrument it is necessary to have the proper high voltage supply and proper impulse amplification and impulse recording circuits; these are mentioned in the last section.

Fig. 5A shows the initial circuit used by Ruthenford and Geiger; it is now of historical interest, but the parallel between it and the succeeding types is quite distinct.

The circuit of Fig. 5B delivers pulses to the amplifier in the following manner: the flight of one ionizing particle through the sensitive volume of the counter tube (or the liberation of a single electron within its confines) immediately initiates the process of collision ionization. Within a time interval of the order of one hundred thousandths of a second the full discharge is formed, and a current flows around the circuit of voltage source, counter, and resistor ( $R$ ). Passage of the minute current through the high ohmage resistor creates a gradient of voltage there (the IR drop) which polarizes the small condenser ( $C$ ) and which then very shortly reaches a value sufficient to cut the potential across the counter tube down below that at which a discharge can be maintained. The discharge in the counter tube is thus "extinguished" by the current flow through the resistor. Thereafter the potential difference between the electrodes of the counter tube is restored while the charge flows off the small condenser toward the ground. During this interval of about one ten-thousandth to one-tenth of a second (depending upon the amount of capacity and resistance in the circuit) a "wave" of electrical polarization has passed over the plates of the small condenser and is impressed upon the amplifier circuit, which ultimately actuates a loud speaker in a sharp "click," flashes a neon tube, or drives an electrical or mechanical recording mechanism.

The circuit of Fig. 5C uses a vacuum tube to extinguish the discharge.<sup>9</sup> After the initial ionizing event, the current starting through the counter tube and  $R_g$  polarizes the control grid,  $G_1$ , which releases a relatively large surge of electricity through the vacuum tube and resistor  $R_p$ ; the current through the vacuum tube produces a large IR drop in  $R_p$ , and thus (in lowering the available voltage) effects rapid extinction of the discharge in the counter tube. Thereafter the charge on the coupling condenser ( $C$ ) is permitted to flow to ground in a short time interval through the relatively small extinguishing resistor,  $R_p$ . The circuit is consequently restored to its initial sensitive state in a very brief interval of time.

<sup>9</sup>G must be maintained at a critical negative value by means of the small battery  $E_g$  for proper operation of the circuit.



The amplifier circuit should be able to receive each of the separate signals from the counter tube to the limit of the counting rate of the Geiger-Müller circuit itself. The amplifier should be so well shielded that it is insensitive to high frequency oscillations in its neighborhood.

Mechanical devices for recording the number of incoming pulses can be used at counting rates of the order 100 to 200 per minute. Near to and beyond this rate the inertia of most mechanical devices is sufficient to cause some loss of the count, or even to cause complete lack of response to pulses coming in at a very great rate. Electrical circuits have much less inertia than mechanical devices and can be used where it is necessary to count at very great speed. Gingrich, Evans, and Edgerton<sup>6</sup> described an arrangement which uses a meter deflection to give the average rate of arrival of the random counter pulses up to rates of several thousand per minute. Neher and Harper,<sup>7</sup> using a circuit due to Hunt,<sup>8</sup> and their own high speed Geiger-Müller circuit, are able to observe at rates up to 200,000 random pulses per minute. Thus it can be seen that Geiger-Müller circuits may be built with tubes and with recording systems adapted to widely different conditions of radiation intensity.

Within the counter tube itself and its immediate circuit, exist factors of central importance to the interpretation of the radiation measurement. Since every single photon and ionizing particle which traverses the sensitive volume does not initiate the discharge process, one speaks of the "efficiency" of the tube: the arbitrary ratio of the number of particles that are intercepted geometrically to the number that are physically effective in discharging the tube.

The efficiency of response of the counter instrument varies with the wave length of the different photons. For this reason the instrument is used essentially to compare the activity of two sources emitting radiations of very nearly the same spectral distribution, and not to make a direct, absolute measurement. Measurements of heterogeneous stray radiations are thus "qualitative" unless they can be compared with a standard source having the same spectrum, or unless the spectral distribution is known and its effect on the counter can be deduced.

The numerical data given by the instrument—as in the regular comparison measurement—are significant and most nearly accurate when they have been corrected for two factors: (a) the finite operation time of the Geiger-Müller circuit (which involves a "counting loss" while the Geiger-Müller circuit is insensitive during the active discharge process), and (b) the statistical fluctuations due to the purely random incidence of the incoming particles. Locher and Weatherwax<sup>9</sup> show how such corrections may be made in a simple manner.

#### REFERENCES

1. Chiewitz, O., and Hevesy, G.: Radioactive Indicators in the Study of Phosphorus Metabolism in Rats, *Nature* 136: 754, 1935.
2. Evans, R. D.: Radium Poisoning. II. The Quantitative Determination of the Radium Content and Radium Elimination Rate of Living Persons, *Am. J. Roentgenol.* 37: 368, 1937.
3. Evans, R. D., and Mugele, R. A.: Increased Gamma-Ray Sensitivity of Tube Counters and the Measurement of the Thorium Content of Ordinary Materials, *Rev. Scient. Instruments* 7: 441, 1937.

\*Hunt, F. V.: *Rev. Scient. Instruments* 6: 43, 1935.

The amplifier circuit should be able to receive each of the separate signals from the counter tube to the limit of the counting rate of the Geiger-Müller circuit itself. The amplifier should be so well shielded that it is insensitive to high frequency oscillations in its neighborhood.

Mechanical devices for recording the number of incoming pulses can be used at counting rates of the order 100 to 200 per minute. Near to and beyond this rate the inertia of most mechanical devices is sufficient to cause some loss of the count, or even to cause complete lack of response to pulses coming in at a very great rate. Electrical circuits have much less inertia than mechanical devices and can be used where it is necessary to count at very great speed. (Hingrich, Evans, and Edgerton<sup>3</sup> described an arrangement which uses a meter deflection to give the average rate of arrival of the random counter pulses up to rates of several thousand per minute. Neher and Harper,<sup>4</sup> using a circuit due to Hunt,<sup>5</sup> and their own high speed Geiger-Müller circuit, are able to observe at rates up to 200,000 random pulses per minute. Thus it can be seen that Geiger-Müller circuits may be built with tubes and with recording systems adapted to widely different conditions of radiation intensity.

Within the counter tube itself and its immediate circuit, exist factors of central importance to the interpretation of the radiation measurement. Since every single photon and ionizing particle which traverses the sensitive volume does not initiate the discharge process, one speaks of the "efficiency" of the tube: the arbitrary ratio of the number of particles that are intercepted geometrically to the number that are physically effective in discharging the tube. The efficiency of response of the counter instrument varies with the wavelength of the different photons. For this reason the instrument is used essentially to compare the activity of two sources emitting radiations of very nearly the same spectral distribution, and not to make a direct, absolute measurement. Measurements of heterogeneous stray radiations are thus "qualitative" unless they can be compared with a standard source having the same spectrum, or unless the spectral distribution is known and its effect on the counter can be deduced.

The numerical data given by the instrument—as in the regular comparison measurement—are significant and most nearly accurate when they have been corrected for two factors: (a) the finite operation time of the Geiger-Müller circuit (which involves a "counting loss" while the Geiger-Müller circuit is insensitive during the active discharge process), and (b) the statistical fluctuations due to the purely random incidence of the incoming particles. Loether and Weatherwax<sup>6</sup> show how such corrections may be made in a simple manner.

#### REFERENCES

1. Chiewitz, O., and Hevesy, G.: Radioactive Indicators in the Study of Phosphorus Metabolism in Rats, *Nature* 136: 754, 1935.
2. Evans, R. D.: Radium Poisoning. II. The Quantitative Determination of the Radium Content and Radium Elimination Rate of Living Persons, *Am. J. Roentgenol.* 37: 368, 1937.
3. Evans, R. D., and Muegler, R. A.: Increased Gamma-Ray Sensitivity of Tube Counters and the Measurement of the Thorium Content of Ordinary Materials, *Rev. Scient. Instruments* 7: 441, 1937.
4. Hunt, F. V.: *Rev. Scient. Instruments* 6: 43, 1935.

The amplifier circuit should be able to receive each of the separate signals from the counter tube to the limit of the counting rate of the Geiger-Müller circuit itself. The amplifier should be so well shielded that it is insensitive to high frequency oscillations in its neighborhood.

Mechanical devices for recording the number of incoming pulses can be used at counting rates of the order 100 to 200 per minute. Near to and beyond this rate the inertia of most mechanical devices is sufficient to cause some loss of the count, or even to cause complete lack of response to pulses coming in at a very great rate. Electrical circuits have much less inertia than mechanical devices and can be used where it is necessary to count at very great speed. Gingrich, Evans, and Edgerton<sup>6</sup> described an arrangement which uses a meter deflection to give the average rate of arrival of the random counter pulses up to rates of several thousand per minute. Neher and Harper,<sup>9</sup> using a circuit due to Hunt,<sup>8</sup> and their own high speed Geiger-Müller circuit, are able to observe at rates up to 200,000 random pulses per minute. Thus it can be seen that Geiger-Müller circuits may be built with tubes and with recording systems adapted to widely different conditions of radiation intensity.

Within the counter tube itself and its immediate circuit, exist factors of central importance to the interpretation of the radiation measurement. Since every single photon and ionizing particle which traverses the sensitive volume does not initiate the discharge process, one speaks of the "efficiency" of the tube: the arbitrary ratio of the number of particles that are intercepted geometrically to the number that are physically effective in discharging the tube.

The efficiency of response of the counter instrument varies with the wave length of the different photons. For this reason the instrument is used essentially to compare the activity of two sources emitting radiations of very nearly the same spectral distribution, and not to make a direct, absolute measurement. Measurements of heterogeneous stray radiations are thus "qualitative" unless they can be compared with a standard source having the same spectrum, or unless the spectral distribution is known and its effect on the counter can be deduced.

The numerical data given by the instrument—as in the regular comparison measurement—are significant and most nearly accurate when they have been corrected for two factors: (a) the finite operation time of the Geiger-Müller circuit (which involves a "counting loss" while the Geiger-Müller circuit is insensitive during the active discharge process), and (b) the statistical fluctuations due to the purely random incidence of the incoming particles. Locher and Weatherwax<sup>5</sup> show how such corrections may be made in a simple manner.

#### REFERENCES

1. Chiewitz, O., and Hevesy, G.: Radioactive Indicators in the Study of Phosphorus Metabolism in Rats, *Nature* 136: 754, 1935.
2. Evans, R. D.: Radium Poisoning. II. The Quantitative Determination of the Radium Content and Radium Elimination Rate of Living Persons, *Am. J. Roentgenol.* 37: 365, 1937.
3. Evans, R. D., and Muehle, R. A.: Increased Gamma-Ray Sensitivity of Tube Counters and the Measurement of the Thorium Content of Ordinary Materials, *Rev. Scient. Instruments* 7: 441, 1937.

<sup>8</sup>Hunt, F. V.: *Rev. Scient. Instruments* 6: 43, 1935.

- 4 Geiger, H., and Muller, W. The Electron Counter Tube, *Phys Zetts* 29 839, 1928
- 5 Gingrich, N. S. Voltage Sources and Amplifiers for Geiger Counters, *Rev Scient Instru-*  
*ments* 7 207, 1936
- 6 Gingrich, N. S., Evans, R. D., and Edgerton, H. E. A Direct Reading Counting Rate  
Meter for Random Pulses, *Rev Scient Instruments* 7 450, 1937
- 7 Hamilton, J. G. The Rates of Absorption of Radio Sodium in Normal Human Subjects,  
*Proc Nat Acad Sci* 23 521, 1937
- 8 Locher, G. L., and Weatherwax, J. L. The Use of Geiger Muller Counters for Locating  
Radium and for Measuring Gamma Ray Intensities, *Radiology* 27 149, 1936
- 9 Ncher, H. A., and Harper, W. W. A High Speed Geiger Counter Circuit, *Physical Rev*  
49 940, 1936
- 10 Rutherford, E., and Geiger, H. An Electrical Method of Counting the Number of Alpha  
Particles From Radioactive Substances, *Proc Roy Soc A* 81 141, 1908
- 11 Street, J. C. and Johnson, T. H. The Use of a Thermionic Tetrode for Voltage Control,  
*J Franklin Inst* 214 157, 1932

## TEST FOR OCCULT BLOOD\*

EDWARD RDOWITZ, M. T., PHILADELPHIA, PA.

IN THE routine examination of specimens for occult blood with benzidine or orthotolidin it is always advisable to have a control in order to check the reagents, particularly in case of a negative reaction because one cannot be sure the reagents are active unless they are checked.

In laboratories where blood is usually on hand it is a simple matter to add a drop of blood to the reagent and observe the reaction immediately. However, when a small laboratory or the general practitioner is making tests for occult blood, it is frequently found necessary to prick the finger in order to obtain blood. Furthermore, if the blood is not diluted sufficiently it does not indicate whether or not a trace of occult blood can be detected. It is necessary, therefore, to make a sufficiently high dilution of the blood before checking the reagent.

The following hemoglobin control solution has been prepared and used for the past year and a half. It is of a high dilution, has remained stable, conserves time, and prevents the danger of laboratory infection from frequent finger puncture.

The control reagent is prepared as follows:

To 20 cc of 50 per cent ethyl alcohol, 10 cc of defibrinated or oxalated blood is added.

Allow to precipitate for thirty minutes, stirring several times during this period.

Filter until clear, through Whatman No. 1 filter paper.

To the clear reddish filtrate, add two parts of distilled water.

A slightly opalescent, light amber solution results.

The reagent will keep indefinitely in a tightly stoppered bottle, a small dropper bottle will be found convenient for this purpose.

One or two drops of this solution will give a strongly positive reaction with benzidine or orthotolidin reagents.

\*From the Clinical Laboratory of Claude P. Brown, M.D., Philadelphia.  
Received for publication March 1, 1938.

# METHEMOGLOBIN DETERMINATION\*

## A CLINICAL METHOD

WILLIAM B. WENDEL, PH.D., MEMPHIS, TENN.

**M**ETHEMOGLOBIN until recently has been a clinical oddity. Its formation as a complication of sulfanilamide therapy makes this nonfunctional derivative of hemoglobin assume wide interest. Speculation rather than analytical data, however, characterizes most statements in the current literature regarding the extent to which accumulation of methemoglobin accounts for the cyanosis and the loss of oxygen combining power of blood of patients receiving sulfanilamide.

The paucity of data on this important side reaction of sulfanilamide appears to be due largely to lack of simple methods for methemoglobin determination. Existing methods require expensive apparatus not found in most hospital laboratories (e.g., spectrophotometer, comparison spectroscope, bicolorimeter) or involve at least one determination of oxygen or carbon monoxide combining power. One relatively simple means of estimating methemoglobin would appear to devolve from determination of oxygen capacity by the Van Slyke method and determination of total pigment by one of the acid hematin methods (Newcomer, Sahli, etc.). If no abnormal pigment other than methemoglobin were present, the difference between total and active pigments should represent methemoglobin. The statement by Peters and Van Slyke,<sup>1</sup> however, that acid hematin methods do not give reliable total pigment values when applied to blood containing methemoglobin and the suggestion of Marshall and Walz<sup>2</sup> that blood of patients receiving sulfanilamide contains aniline black must have deterred some from using this method. According to our experience, the Newcomer method gives reliable total pigment values on blood containing methemoglobin and we find no evidence that aniline black is present in the blood of sulfanilamide treated patients. Spectrophotometric measurements which will be published shortly indicate that, except in a small number of cases where sulfhemoglobin has been observed, methemoglobin is the only abnormal pigment likely to be encountered. We, therefore, consider the total pigment-oxygen capacity method suitable for clinical determination of methemoglobin.

We have examined several hundred specimens of blood from patients receiving sulfanilamide and have found methemoglobin in every specimen which contained more than 4 mg. per cent of sulfanilamide. Failure of others to detect methemoglobin is certainly due in some instances to use of the inadequate qualitative tests which are found in most textbooks of biochemistry and handbooks

\*From the Departments of Internal Medicine and Biological Chemistry, Washington University Medical School, St. Louis, Mo.

Received for publication, May 14, 1933.

of clinical laboratory procedures. The statement so frequently encountered, that methemoglobin cannot be detected spectroscopically in blood unless it constitutes 20 to 30 per cent of the total pigment is untrue. Methemoglobin can be detected spectroscopically in blood when it represents as little as 3 per cent of the total pigment.

A rapid and simple procedure for quantitative determination of methemoglobin is described below. The method requires special apparatus, but of an inexpensive nature. A determination can be carried out in less than five minutes if the fraction of blood pigment present as methemoglobin alone is desired. A knowledge of only the elements of spectroscopy is required of the analyst.

The determination depends upon the following facts: (a) Methemoglobin has a characteristic absorption band in the red portion of the visible spectrum at  $\lambda = 630 \text{ m}\mu$ . (b) The width and the intensity of this absorption band are proportional to the concentration of methemoglobin. (c) Oxymethemoglobin as compared with methemoglobin absorbs little light in the red region of the spectrum.

In practice all of the pigment in one sample of a specimen of blood is converted into methemoglobin and the methemoglobin concentration of this is compared with that of an untreated sample of the same specimen. This comparison is accomplished by diluting the two samples until they have the same methemoglobin concentration, as determined by the width and the intensity of the characteristic absorption band. The ratio of these two dilutions represents the fraction of total pigment present in the form of methemoglobin.

#### APPARATUS

1. A Schmidt and Haensch hand spectroscope (*S* in Fig. 1) with adjustable slit and long focus magnifier is recommended because of its clear optical field and high dispersion\*. This spectroscope has two moving parts, the telescope and the slit, the functions of which must be understood by the user. The telescope is for focussing on the spectrum and should be extended until the Fraunhofer lines (dark lines crossing the spectrum when the spectroscope is pointed toward the sky and the slit is nearly closed) are sharp. The slit determines the width of the pencil of light impinging upon the prism and thus the purity of the spectrum obtained. Maximum sensitivity in spectroscopic work is obtained when the slit is as nearly closed as is compatible with good visibility.

2. The specimen tubes (*ST* and *U*) are 100 c.c. graduated cylinders, the flared bottoms of which have been ground down parallel with the side walls. These should not be completely encircled by graduations, except perhaps at the 10 c.c. intervals, and should have the same diameter between the 5 and 15 c.c. calibration. This is checked by measuring the longitudinal distance between the 5 and 15 c.c. calibrations. Cylinders which differ by not more than 0.5 mm are satisfactory.

\*Hand spectroscope No. 221a and 221a Akatos Inc. New York N. Y. These two spectroscopes are identical except that 221a has accessories (a comparison prism and illumination mirror) which although not required for methemoglobin determination may be useful if the instrument is put to other use. See Harrison's *Methods in Clinical Medicine*, Macmillan 1930 for brief discussion of the hand spectroscope.

3. The movable holder for the cylinders is a block of wood (dimensions,  $3\frac{1}{2}$  inches long,  $2\frac{1}{4}$  inches wide, and  $3\frac{1}{4}$  inches high) into or through which four holes are bored. The two vertical holes are for the cylinders and have a diameter  $\frac{1}{8}$  inch greater than the outside diameter of these. They are about 2 inches deep. Thin felt strips glued to the inner walls of these holes keep the cylinders in position when, in the course of comparing the spectra of the two solutions, the block is moved *from side to side*. The two horizontal holes ( $\frac{1}{2}$  inch in diameter), which pass through the block at the level of the 15 c.e. calibration of the cylinders, permit a beam of light to pass through the solution in the cylinder and into the spectroscope. If the level of the solution in the cylinder does not stand above the beam of light, the cylinder is raised off the bottom and supported by a cork.

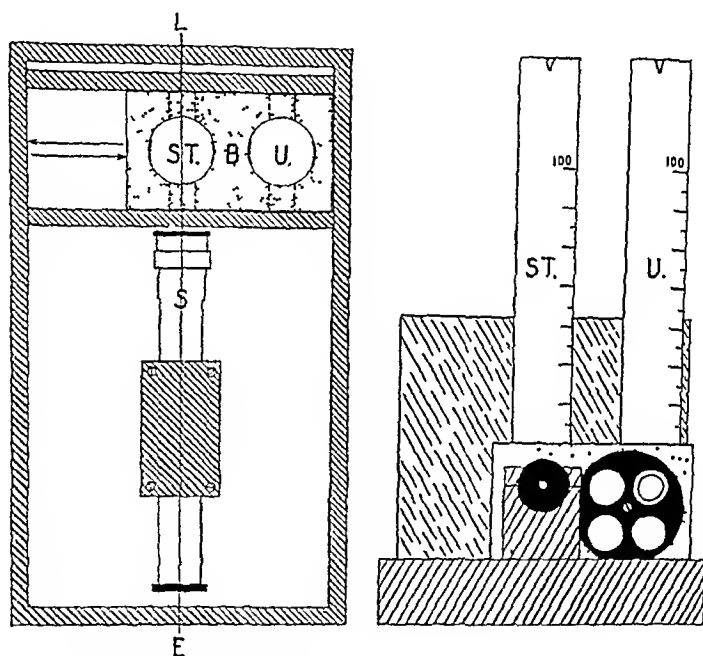


Fig 1.—Apparatus for methemoglobin determination

4. A red glass optical filter,\* 3 mm. thick and about 20 mm. in diameter, is fixed on the cylinder block in front of the standard. A metal disk carrying three red filters, 3 mm., 2 mm., and 1 mm. thick, respectively, and having an additional hole not covered by a filter, is attached to the cylinder block before the unknown in such a manner that any one of the filters or no filter may be brought into the path of light passing through the solution. A single filter, 1 or 2 mm. thick, attached to the end of the spectroscope, can be used instead of the four, but with some sacrifice of convenience and accuracy.

5. The base of the apparatus, the holder for the spectroscope, the shield, and a cover (not shown in the illustration) are made of wood. A 60 watt bulb placed 2 or 3 inches behind a hole in the shield serves as a source of bright

\*The Iowa Colored Optical Glass Filter, RG-1, is recommended. It may be obtained from the Fish-Schurman Corporation, 230 East 45th Street, New York, N. Y., in 2 inch squares. The required sizes and thicknesses can be cut and ground from one such square of 3 mm. thickness.

light The base shown in the illustration is attached to a second base upon which the light is fixed in order that the alignment of the spectroscope and the light will not be disturbed in the course of the determination

#### REAGENTS

(1) 0.05 per cent Saponin solution Use only a pure white powder which gives a perfectly clear solution Preserve with toluene

(2) 30 per cent Potassium ferricyanide solution

(3) 20 per cent Ammonium acetate solution Use only the reagent quality which has been kept tightly stoppered This solution after diluting 1:20 should be pH  $7.0 \pm 0.2$

#### PROCEDURE

Measure 1 cc of blood into one of the cylinders Lave for one minute with 4 or 5 cc of saponin solution and add 1 cc of the ammonium acetate solution\* Examine with the spectroscope for the presence of methemoglobin as follows Starting with the slit of the spectroscope closed, gradually open it until red light is seen If the blood contains methemoglobin in quantities greater than 3 to 5 per cent of the total pigment, a dark band will be seen across the red region of the spectrum (This absorption band of methemoglobin disappears if the solution is made alkaline with sodium carbonate or sodium cyanide The somewhat similar band of sulfhemoglobin is not so affected) If the concentration of methemoglobin is very high, the field may be so dark that the methemoglobin band will not be visible until the blood is further diluted If more than a trace of methemoglobin is present prepare the standard methemoglobin solution as follows Measure 0.5 cc to 1.0 cc of blood into another cylinder containing about 10 cc of saponin solution and after laving is complete (one minute), add 1 cc of ammonium acetate solution and 1 or 2 drops of potassium ferricyanide solution Dilute further with saponin solution until the methemoglobin band is greatly decreased in intensity An appropriate dilution of the standard is 1:100 if the total pigment concentration of the specimen is approximately normal A proportionately smaller dilution is required with anemic bloods The standard methemoglobin solution is now placed in the cylinder holder between the light and the spectroscope, and the slit of the spectroscope is adjusted until a moderately bright field is obtained Without changing the slit the spectra of the two solutions are compared by rapidly moving the block from side to side If the width and the intensity of the absorption bands of the standard and the unknown differ, the latter solution is diluted with saponin until the two are alike—or if the standard is stronger, it is diluted The thickness of the filter used before the “unknown” should be increased as the dilution becomes greater At low dilutions no filter is placed before the unknown The thickness of the filter to be used at a given dilution is that which makes the orange yellow portion of the spectrum between the methemoglobin band and the filter boundary alike as to width and intensity in the spectra of the two solutions The dilu-

\*The ammonium acetate provides a buffered solution required because methemoglobin is an acid base of the acid form which is used in the determination in the acid form and the oxygenation of the hemoglobin

The buffer spectrum of hemoglobin is



tions at which the absorption bands of methemoglobin in the two solutions are alike as to width and intensity are noted. From these figures the fraction of pigment present in the blood in the form of methemoglobin is calculated. If absolute concentration of methemoglobin is desired, total pigment also is determined by a method which depends upon the formation of acid hematin (Newcomer, Sahli, etc.) or cyanmethemoglobin (Stadie, Wu) or by iron analysis (Kennedy, Wong). Colorimetric methods depending upon carbon monoxide (Palmer) and methods depending upon oxygen capacity (Van Slyke) cannot be used.

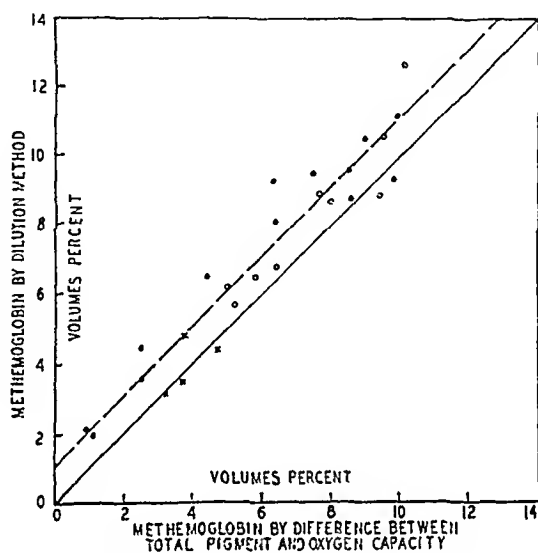


Fig. 2.—Comparison of results of methemoglobin determination by two methods. (The three different kinds of designations represent observations of three different individuals.)

#### CALCULATION

##### 1. Percentage methemoglobin:

$$\frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} \times 100 = \text{Methemoglobin, as per cent of total pigment.}$$

##### 2. Absolute methemoglobin concentration:

$$\frac{\text{Dilution of Unknown}}{\text{Dilution of Standard}} \times \text{Total Pigment} = \text{Methemoglobin in grams per 100 c.c. of blood, or volumes per cent, depending upon the choice of terms of expressing total pigment.}$$

*Example:* Standard, 0.5 c.c. of blood diluted to 40 c.c.; unknown, 1.0 c.c. of blood diluted to 20 c.c. Methemoglobin =  $\frac{20}{80} \times 100$ , or 25 per cent of the total pigment.

#### DISCUSSION

A comparison of the concentrations of methemoglobin in 26 samples of blood (from patients receiving sulfanilamide and dogs receiving nitrite) by the simple dilution method described here and the total pigment-oxygen capacity method shows remarkably good agreement. Total pigment was determined in most instances as acid hematin by the Newcomer method, in some as cyanmethemoglobin, spectrophotometrically. In Fig. 2 it is seen that the dilution method gives values for methemoglobin which are higher by about one volume per cent. This is true irrespective of the total pigment or methemoglobin concen-

tration. The broken diagonal line represents the mean deviation. The average deviation from this mean is only 0.6 volumes per cent. Most of these observations were made with a single filter of 3 mm thickness and since it has been our experience that the subjective aspects of the method are improved by the use of filters of different thicknesses, it is probable that the method as described gives even better values. In any case it is adequate for clinical purposes.

Wu<sup>3</sup> and Stadie<sup>4</sup> state that acid hematin methods do not give reliable total pigment values when applied to blood containing methemoglobin. Both workers apparently used blood to which a large excess of ferrievanide had been added. Our experiments show that the Newcomer procedure gives correct total pigment values on bloods containing methemoglobin produced by nitrite.

#### SUMMARY

A simple, rapid, direct spectroscopic method for quantitative determination of methemoglobin in blood is described.

*Addendum.* A microadaptation of the above procedure using one fifth as much blood in standard and unknown and proportionately smaller amounts of the several reagents, and performing the dilutions in 10 cc instead of 100 cc graduated cylinders permits determination of methemoglobin on finger tip blood. For standard 0.1 cc of blood is diluted to 2 to 4 cc, depending upon the total blood pigment concentration.

Further study of the method has shown that smaller hand spectrosopes than the ones recommended can be used without significant loss of accuracy.

The valuable assistance of Miss Jean Fletcher is acknowledged.

#### REFERENCES

1. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry, Baltimore, 1932, Williams & Wilkins Co., Vol. II, p. 663.
2. Marshall, E. K., and Walzel, E. M. On Cyanosis From Sulfanilamide, Bull. Johns Hopkins Hosp. 61, 140, 1937.
3. Wu, H. Studies on Hemoglobin. I. The Advantage of Alkaline Solutions for Colorimetric Determination of Hemoglobin, J. Biochem. 2, 173, 1923.
4. Stadie, W. C. A Method for the Determination of Methemoglobin in Blood, J. Biol. Chem. 41, 237, 1920.

## A NEW BABY BALANCE

ERICH LOEWENSTEIN, PH.D., FLUSHING, N. Y.

I WISH to describe a new baby balance which is used mostly in children's hospitals for the purpose of measuring the insensible perspiration. The insensible perspiration is an important physiologic function, providing one of the three avenues for loss of heat. When environmental conditions are reasonably constant, about 25 per cent of the total calories are lost by the evaporation of water from the body. Thus from the weight loss, the basal metabolic rate can be predicted. It has been shown by Levine and others that the prediction is sufficiently accurate for most clinical purposes. The method is particularly useful in the case of children, who, because of inability to cooperate, cannot be measured in the conventional basal metabolism apparatus. The sensitivity of the Sartorius baby balance has made it possible to introduce a damping device. Thus, since shorter periods can be used for weighings, a closer correlation can be maintained between loss of weight and extraneous factors which modify the metabolism. The balance can also be used for many other purposes, such as the calibration of volumetric flasks, where a high sensitivity is required with a large capacity. It can also be used for recording the growth of plants or animals, and for the insensible perspiration of animals. The accuracy of this balance is 10 mg. at a capacity of 25 kg.

The balance possesses several distinctive features: It is fitted with specially constructed suspensions (1) which compensate for the pendulum motion of the balance pans (2). This type of suspension compensates for oscillations in all directions.

It is equipped with circular arrestment (3). This arrestment disengages the three special steel knife edges (4) in such a way that friction across the knife edges cannot occur, a feature which results in an unaltered sensitivity for a long period of time. The arrestment may be equipped either with a wheel or, for smoother release, with a lever.

In place of the old type free swinging beam, the new balance has a simple oil-damping attachment (5) which brings the beam to rest after a single swing. A paddle is fixed to the beam in such a way that as the beam swings, the damping effect of the oil is brought to bear on the paddle. The use of a magnetic or electromagnetic type of damping was avoided since the accuracy of a balance can be affected by a magnetic field. The pointer (6) carries a photomicrographic scale (7), which by means of the projection device appears illuminated on a ground glass screen (8) against a hair line. The new projection device is fixed to the pillar to prevent zero point changes.

Received for publication, July 20, 1937.

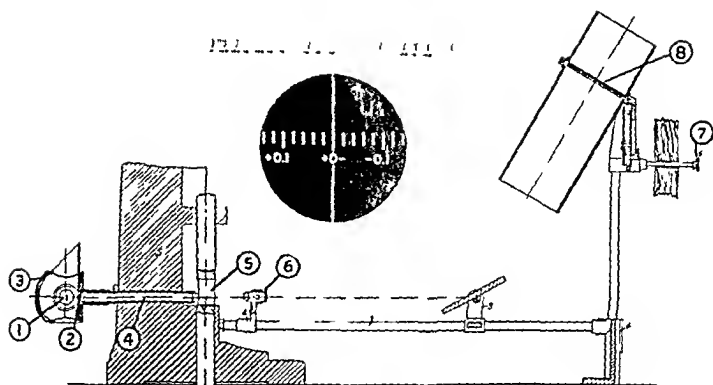


Fig 1

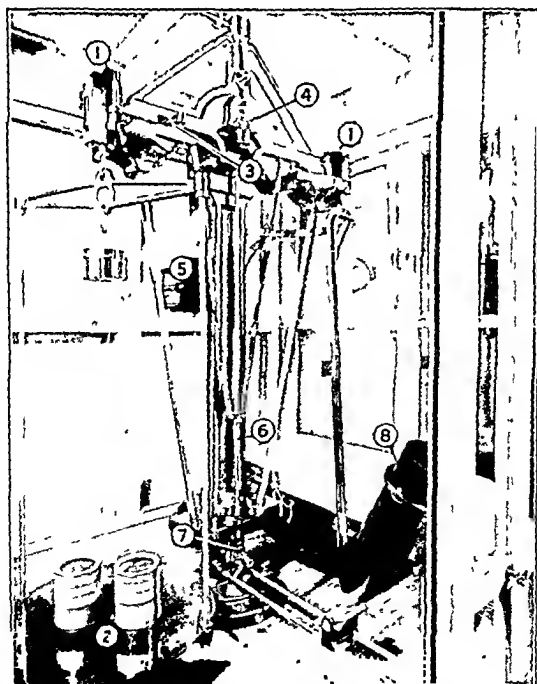


Fig 2

The new projection apparatus operates as follows:

An electric bulb (1) (see Fig. 2) of 10 to 12 volts mounted in a socket (2) serves as a source of light. The lamp housing (3) is covered entirely so that the infants will not be disturbed by the light. The electric current is drawn from the main through a transformer or resistance and thence to an automatic contact switch. This switch is operated by the knob for the combined pan and beam release. An alarm signal indicates that the arrestment of the balance has been released, and serves as a warning that the infant should not be removed at this time. Damage to the knife edges is thereby prevented. The light from the bulb is carried through a tube (4) which contains the condensing lenses. The light then passes through an optical scale (5) and through an objective (6). The objective permits adjustment of the sharpness of the image on the screen (8). A fine hair line permits adjustment of the zero point. This zero point adjustment is easily accomplished from outside the case by a screw (7). The device permits the reading of masses from 0.01 gm. to 1.0 gm. without the use of fractional weights. The scale is graduated on both sides into 50 divisions, each division being equal to 0.02 gm. By estimation between the divisions 0.01 gm. can easily be read. The figures read from 0.1 to 1.0 gm. and are designated as plus or minus, indicating that the scale reading is either to be added to, or subtracted from the final weight. The divisions and numbers on the scale appear sharply defined on a dark background and easily permit accurate readings from a distance. Therefore, a special table for the balance is not needed. These features allow one to make weighings with great rapidity. Errors associated with the handling and reading of weights and a rider, and from occluded dirt, etc., are entirely eliminated. The large projection of the scale on the screen permits accurate reading at a long distance without eyestrain.

The beam is of the short armed type (24 inches) and is protected with a black acid-proof varnish. The knife edges are of special hard steel, and the plates of fine agate. The diameter of the pan on the left side is about 15 inches. The pan holder is covered with acid-proof varnish, and has a height of 30 inches, and a width of about 15 inches. On the right side a special aluminum basket is mounted on the pan (length 40 inches, width 14 inches). This basket is covered with a net so that the infants do not come in contact with cold metal. Netting is also provided to prevent the babies from touching and disturbing the mechanism of the balance. The balance is completely enclosed in an oak housing with front and side doors. The dimensions of the housing are as follows: 56 inches height, 52 inches width, and 48 inches depth. The balance is mounted on a heavy oak base plate with leveling screws, and level on the base of the pillar.

To further simplify and speed up the weighing procedure, the balance can be equipped with a device for manipulation of weights from 1 to 100 gm. from outside the case. The device is operated by means of a double knob arrangement, consisting of an inner dial and outer ring. The total of these accessory weights is indicated by an arrow. By turning the inner dial, weights from 10 to 90 gm. can be added, and by turning the outer ring, weights from 1 to 9

gm can be put on the auxiliary beam. The weights, which are placed on this notched beam over the left hanger, are horseshoe shaped and are suspended from long hooks which when raised or lowered put on or take off the weights. The weights so arranged will withstand severe jarring without falling from their suspension. This type of weight manipulation operates easily and eliminates much of the labor of adding single weights to the weight pan. The speed of manipulation of weights by turning the knobs is at least five times faster than handling individual weights with forceps. In addition, since the weights are not handled, they keep their accuracy, and are free from dust.

When weighings are to be made over a long period, say from one to two hours, it is perhaps advisable to provide curtains around the outside of the case so that the infant will not be disturbed.

In order to have reproducible conditions of temperature and humidity, a thermograph and hygrograph may be installed in the room. These instruments can be fitted with contacts which actuate relays and operate an air conditioning apparatus. The temperature of the baby can be measured by rectal thermocouple or by an electric resistance thermometer.

The results obtained with this balance are exceedingly satisfactory. The zero point has proved constant, the sensitivity is high, the damping device is effective, and the projection reading device is convenient. If for special purposes it is desirable to have a recording device instead of a projection reading device, a simple photomicrographic recording attachment can be furnished with the balance.

The author is deeply indebted to Pfaltz & Bauer, Inc., New York, who manufactured and installed this balance for their invaluable aid in the development of this apparatus.

#### REFERENCES

- Levine, Samuel Z., Wilson, James R. and Kelly, Margaret. The Insensible Perspiration in Infancy and in Childhood. *Am J Dis Child* 39: 917, 1930.
- Levine, Samuel Z., and Wyatt, T. C. The Insensible Perspiration in Infancy and in Childhood. *Am J Dis Child* 40: 269, 1930.
- Levine, Samuel Z., and Wyatt, T. C. The Insensible Perspiration in Infancy and in Childhood. *Am J Dis Child* 44: 732, 1932.
- Levine, Samuel Z., and Wheatley, M. A. Respiratory Metabolism in Infancy and in Childhood. *Am J Dis Child* 51: 1300, 1936.
- Nickerson, R. F. An Improved Technique for Determination of Insensible Perspiration. *J LAB & CLIN MED* 22: 412, 1937.
- Ginandes, G. J., and Topper, A. Insensible Perspiration in Children. *Am J Dis Child* 52: 1335, 1937.
- Ginandes, G. J., and Topper, A. Insensible Perspiration in Children. *Am J Dis Child* 52: 528, 1936.
- Ginandes, G. J., and Topper, A. Insensible Perspiration in Children. *Am J Dis Child* 53: 765, 1937.
- Johnston, M. W., and Newburgh, L. H. *J Clin Investigation* 8: 147, 1929.
- Benedict, F. G., and Root, H. F. Insensible Perspiration: Its Relation to Human Physiology and Pathology. *Arch Int Med* 38: 1, 1926.
- Benedict, F. G., and Wardlaw, H. S. H. Some Factors Determining the Insensible Perspiration of Man. *Arch Int Med* 49: 1019, 1932.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

---

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

---

**STREPTOCOCCI, Hemolytic, Bacteriological and Clinical Study of Professional Personnel of Maternity Hospitals, With Special Reference to Carriers of, Bryce, L. M., and Tewsley, P. Med. J. Australia 1: 639, 1938.**

A brief description is given of an outbreak of a puerperal fever, involving three patients in a maternity hospital. The measures adopted to deal with it and those designed to prevent future similar outbreaks are outlined.

The value and limitations of periodic swabbing of the nose and throat of midwifery workers are discussed.

The bacteriologic technique is described. In addition to the recognized biochemical and serologic methods of classification of hemolytic streptococci, their sensitivity to four serologically distinct types of streptococcal bacteriophage was tested; it was not found to be in complete agreement with the results of the precipitin test. The variation of sensitivity to bacteriophage among strains falling into Lancefield's group A suggests, however, that this property might be used in epidemiologic studies.

The incidence of throat carriers of group A streptococci among the contacts of the three patients with puerperal fever was found to be 22 per cent. On two subsequent occasions, when there had not been recent known contact with streptococcal infections, the incidence of group A streptococci among similar groups of medical practitioners and nurses was 3 per cent and 6.7 per cent, respectively.

Cases are described and tables are given illustrative of the varying problems encountered in different individuals.

In persons with healthy respiratory tracts the presence of hemolytic streptococci in throat swabs appeared to be closely associated with recent contact with streptococcal illness. In those with clinically detectable abnormality of the nasopharynx there was a greater tendency to harbor hemolytic streptococci, irrespective of recent contact. It is, therefore, suggested that exclusion from midwifery units of persons with such abnormality, by means of preliminary medical examination, would be a possible way of reducing the risk of infection of patients and the disorganization attendant on their withdrawal during a period of midwifery work.

**POSTMORTEM EXAMINATION of Stillborn and Newly Born Infants, Potter, E. L. Arch. Path. 25: 608, 1938.**

The post-mortem examination of the stillborn infant and the infant dying in the neonatal period presents certain differences from post-mortem examination of the adult. The causes of death in the stillborn and the liveborn infant are in general similar and are largely related to ante-partum and intra-partum obstetric complications. There is a small group of infants who succumb because of abnormalities of the ova or spermatozoa have prevented normal development, and there is a small group who die from infections. Most of those remaining die from abnormal factors associated with the placenta, umbilical cord, birth processes, or the establishment of extrauterine life.

To determine the cause of death is frequently difficult, and if the body of the infant is the only material available, it is frequently impossible. To make it possible to arrive at a reliable conclusion, an adequate clinical history must be available, and the placenta, cord, and infant must be examined. Autopsy of the infant should invariably include examination of all parts of the body, including the cranial, thoracic, and abdominal cavities, the neck, the vertebral column, and the long bones. Microscopic examination of the viscera is essential.

**JAUNDICE, Nature of the Bleeding in, Quilek A J J V M V 110 1668 1938**

Prothrombin deficiency is an important cause of defective and prolonged coagulation of the blood. A wide margin of safety, however, allows nearly 80 per cent of the prothrombin in human blood to be lost before serious hemorrhage occurs. In various types of jaundice the prothrombin may drop to an exceedingly low level and it has been found that the bleeding tendency corresponds to the prothrombin depletion. Experimentally it has been demonstrated that the prothrombin can be reduced by deprivation of vitamin K, by a toxin such as occurs in spoiled sweet clover hay, and by an injury to the liver. In all types of prothrombin deficiency, blood transfusion is a prompt therapeutic means of stopping bleeding, since sufficient prothrombin is supplied to assure normal coagulation, but its beneficial action is temporary. The permanent cure depends on restoration of normal hepatic function. A vitamin K deficiency as a cause of hemorrhage has not been demonstrated clinically but it is probable that it may be a cause of bleeding in man.

**TISSUE "Inflammatory Carcinoma" of the Breast Taylor, G W, and Meltzer, A Am J Cancer 33 37, 1938**

A clinical study is presented of 8 cases of inflammatory carcinoma of the breast seen at Pondville Hospital over a nine and one half year period.

This grave disease is not rare. The literature contains over a hundred cases. The incidence was 4 per cent of all breast cancers in our series.

Although the disease is rare after seventy its age distribution is the same as for cancer of the breast in general.

The inflammatory signs may arise simultaneously with the cancer (primary type) or they may occur after a scirrhous cancer has been present for some time (secondary type). Pain is a common early symptom in the primary group. Inflammatory signs may result in mistakes in diagnosis and injudicious early therapy.

The primary cases come to medical attention early, yet their inflammatory signs are usually fall blown and the disease is widespread on admission. The cancer may have an acute erysipeloid distribution or show a tendency to nodular localization. Ulceration is rare.

The disease spreads rapidly in the superficial lymphatic structures of the chest wall. Multiple visceral metastases occur early, but the rapid course of the disease often does not permit them to attain clinical recognition. Bone metastases were recognized roentgenologically in only 4 primary cases. In the uncomplicated cases, leucocytosis, fever, and other signs of toxicity are rare. The patients maintain remarkably good health through the greater part of the course and cachexia is unusual. Death is most often due to intrathoracic complications. The average duration of life in primary cases was 213 months, in secondary cases 105 months after the appearance of inflammatory signs.

The inflammatory signs of edema, redness, and heat are due to extensive lymphatic blockage by cancer and congestion of the subpapillary plexus. There is no uniform pathologic type.

The large fatty breast seems a predisposing factor as does the hyperplastic breast of late pregnancy or lactation. No other predisposing factors could be established.

The results of therapy are poor. Surgery is followed by prompt evidence of supraclavicular disease, skin recurrence, or invasion of the opposite breast. X-ray seems to give the best palliative results. Artificial menopause does not alter the course of the disease.

**GINGIVITIS, Acute Infectious, Black W C Am J Dis Child 56 126, 1938**

The term "acute infectious gingivostomatitis" is proposed for that common but widely misunderstood and mistreated disease of the mouth in children which is characterized by marked gingivitis, oral fetor, fever, and enlargement of the regional lymph nodes. There are serious objections to the use of any one of the numerous terms now applied



to this disease. Its cause is unknown. It is unlikely that the oral anaerobic organisms, among which are Vincent's fusiform bacillus and spirillum, play more than a subordinate role in the pathogenesis. Oral anaerobic organisms of various sorts are constant inhabitants of the anaerobic crevices of the normal mouth, even in young children. They may temporarily increase in number during any gingival inflammation. Individually and collectively they are practically bereft of pathogenicity for laboratory animals.

By clinical and laboratory analysis in 69 cases, it has been found that acute infectious gingivostomatitis affects the hardy and well-kept child as commonly as it does the malnourished and dirty. It occurs most commonly in the fall and winter months, and the incidence is about twice as high in girls as in boys. The peak of age incidence comes at thirty months, while in four-fifths of all cases the disease occurs before the fourth birthday. Fever, the symptom most commonly present at onset, often precedes the appearance of local lesions by one to three days. The gingivitis and oral feter frequently reach their peak of intensity after the constitutional symptoms have subsided. More than half of the patients have little round, shallow, gray oral or labial ulcers. Of the patients in which these ulcers do not occur, about five times as many are girls as boys, while the incidence of ulcers shows no significant sex difference. The disease, from the appearance of the first symptom to complete healing, usually lasts from nine to fifteen days.

The disease is benign and self limited. Recurrence and second attacks are rare. No important complications were encountered in the present series. The affliction is mildly contagious; the principal danger is that infection may occur in young children who come into intimate contact with a patient. Debilitated children or children suffering from measles should be rigidly protected from exposure to this disease.

**RHEUMATIC FEVER, Serum Cholesterol in Patients With, Offenkrantz, F. M. Am. J. Dis. Child. 56: 68, 1938.**

A complete series of 100 patients with rheumatic fever has been reported on. Forty-nine of these were taken as a control group, since they had no evidence of rheumatic activity. Evidence of such activity consisted of elevation of the leucocyte count above 10,000 per c.mm.: an erythrocyte sedimentation rate above 12 mm. per minute, and increasing cardiac damage, as shown by electrocardiographic study. The mean total value of the serum cholesterol (and standard deviation) was  $181.9 \pm 23.5$  mg. per 100 c.c.; the mean value of the free serum cholesterol was  $50.5 \pm 7.4$  mg.; the mean percentage of free cholesterol was  $27.3 \pm 2.23$ .

Of the remaining 51 patients, 5 died. Analyses of serum cholesterol within one day prior to or ten minutes after death revealed severe depletion of the cholesterol ester and only slight lowering of the free cholesterol level, so that the percentage of free cholesterol rose above 30 in each instance.

Eleven case histories were presented of children who seemed to suffer activity of the rheumatic fever throughout the period of observation. There was no cardiac failure of the right or left side, and all patients had negative Schick and Mantoux reactions. Here likewise the total cholesterol levels of the serum seemed to be definitely lowered and the percentage of free cholesterol elevated, so that the depletion was ascribed to the ester fraction.

In the next series (12 cases), the status of the disease fluctuated, periods of rheumatic inactivity being interrupted by flare-ups. The periods of activity again showed the lowered serum cholesterol level with the elevated percentage of free cholesterol (loss of ester). A general inverse relation was noted between the severity of the ester depletion (with a rise in the percentage of free cholesterol) and the sedimentation time, but this relation was not quantitative.

In 9 patients periods of cardiac decompensation were associated with the rheumatic infection. During such periods of failure, with passive congestion of the liver, the flare-up failed to produce rise in the percentage of free cholesterol, although there was a moderate

fall in the total cholesterol values. This indicated that both the ester and the free cholesterol content were lowered. A similar situation seemed to prevail in the 7 cases in which a fluctuating status of disease was complicated by a positive Mantoux reaction, though in 6 of these cases there was no clinical or roentgen evidence of active tuberculosis. Lastly, 5 cases were cited in which unusually high values for total cholesterol prevailed, despite activity of the disease. It was suggested at this point that possibly a psychiatric-functional status is associated with a particular type of cholesterol level in the blood.

The pertinent literature for this study is cited, and the salient findings are discussed.

**BENZEDRINE, Color Reaction for, Richter, D. Brit. Lancet 1: 1275, 1938**

Using the micromethod described below, it was found that with doses of 20 mg. benzedrine sulfate in some subjects only 30 to 50 per cent is excreted in the urine in twenty-four hours, and benzedrine may still be detected in the urine forty hours after administration.

*Benzedrine gives a yellow color with a number of nitrophenols, including picric acid, and this may be used as a convenient method of estimation for amounts down to 0.001 mg. per c.c.*

To 60 c.c. urine in a 250 c.c. separating funnel, are added 6 c.c. petroleum ether and 4 c.c. 2 N caustic soda solution. The mixture is shaken three minutes by a rotating motion to prevent froth formation, allowed to stand two minutes, and the aqueous layer run off. The petroleum ether layer is centrifuged five minutes, cleared by stirring with a glass rod, and centrifuged for a further two minutes. Three cubic centimeters of the petroleum ether solution is measured in a dry pipette or 10 c.c. measuring cylinder and washed with 3 c.c. chloroform into a dry test tube; 0.6 c.c. of a solution containing 1 per cent picric acid in toluene is now added. The tube is allowed to stand well corked for twelve hours, during which time the picrates of other basic substances from the urine separate out; the clear yellow solution is then compared with a series of standards prepared in a similar manner from solutions containing 0, 1, 2, 5, 10, and 20  $\gamma$  benzedrine per c.c. For making the standards pure liquid benzedrine was used and the solution titrated with standard N/5 acid, but for many purposes it is sufficiently accurate to use a solution obtained by heating a 10 mg. tablet of benzedrine sulfate with 7.35 c.c. water (the starch with which the tablets are made up does not interfere). The resulting solution contains 1 mg. base per c.c.

Normal urines often contain small amounts of amines which give a slight blank value corresponding to about 0.5 to 1.0  $\gamma$  benzedrine per c.c. The value of the blank must, therefore, be determined and subtracted from the values obtained after giving benzedrine. Small amounts of indol in the urine do not interfere with the estimation, but the formation of amines by bacterial decomposition must be avoided by adding a few drops of toluene and using the urines as fresh as possible. Traces of moisture or alkali in the tubes interfere with the estimation, and it is essential that the tubes should be well cleaned with cleaning fluid and dried.

**SPINAL FLUID, a Globulin Test for, Newman, K. O. Brit. Lancet 1: 1333, 1938.**

The method suggested is considered superior to Pandey's and consists in using a 5 per cent solution of pure tannic acid in distilled water as a reagent, instead of phenol. The solution is rapidly made and keeps well. It is innocuous and can be prepared without laboratory equipment. The sensitivity of the test is very slightly greater than Pandey's, and the result of it can still be seen and estimated, after a considerable period, whereas the result of Pandey's test vanishes after a few minutes.

The test is best carried out by putting approximately 1 c.c. of 5 per cent tannic acid into a watch glass. One drop of cerebrospinal fluid is allowed to run into the solution, and the reaction reaches its maximum within a minute, after which it remains stationary. A positive reaction is graded in the usual way, as + or ++, or +++.

**LIVER, Fatty Infiltration of, and the Development of Cirrhosis in Diabetes and Chronic Alcoholism, Connor, C. L. Am. J. Path. 14: 347, 1938.**

Fatty infiltration of the liver occurs in those conditions where, because of lack of intake or absorption of food, fat is mobilized from the existing fat depots; and where, because of internal interference with the metabolism of fat due to anoxemia or tissue anoxia, the accumulated fat cannot be broken down for use. In the first instance it results from external starvation; in the second, from what may be called internal or tissue starvation. In both instances normal carbohydrate-fat metabolism does not take place. Among the conditions in which this normal metabolism is altered or inhibited are the various diseases which, by their nature, are called wasting; the disease diabetes, and the disease following the introduction of poisons which inhibit proper tissue oxidation, the most common of which is alcohol. Other diseases occur but are not dealt with here.

In the starvation accompanying progressive morbid states the condition is of little pathologic importance, being in most cases terminal in nature. In diabetes the enlarged fatty liver may be influenced by insulin and by strict dieting, but also may persist for many years. In alcoholism a variety of factors operate to produce the same condition. Alcohol alone will cause some fatty infiltration, but as relative and sometimes absolute starvation is constantly associated with severe chronic alcoholism, the development of fatty infiltration of the liver most often depends on a combination of these two. The absence of vitamin B<sub>1</sub> in the diet may contribute also to this, but such deficiency is probably of minor importance.

Experiments cited in this paper, and analysis of existing recorded observations and experiments, indicate that a liver containing demonstrable neutral fat is in most cases pathologic; that fat may pass into and out of the liver with astonishing rapidity; and that fat may be present in such amounts as to interfere seriously with both the metabolic and the mechanical functions of the organ. In two cases of diabetes this chronic fatty infiltration went on to portal cirrhosis. One of these cases was so severe that the patient died of hemorrhage from esophageal varices. The development of perilobular fibrosis seems to be the result of a combination of mechanical pressure, local tissue anoxia, and general anoxemia, causing atrophy and degeneration of liver cells.

The alcoholic liver occurs in two forms, one of which is the precursor of the other. The first is the enlarged tense liver so swollen with fat that the distended surface lobules present the appearance of cirrhosis. This effect is further simulated by the intrahepatic block, which interferes with excretion of bile, and the transmission of portal blood. The clinical signs of jaundice and ascites are thus produced. Many such patients lapse into coma and die, and at autopsy the large liver is the only prominent finding. These show lobules distended with fat, and some show, in addition, hyaline degeneration and atrophy of peripheral cells associated with early proliferation of fibroblasts. A series of cases presented here represents this group, and another series illustrates the second form in which further proliferation of connective tissue produces the typical picture of portal cirrhosis as seen in chronic alcoholism. An unmistakable gradation of the one into the other is so manifest that the mechanism of the production of alcoholic cirrhosis seems to me to have been demonstrated. The absence of fat in many such livers at the end is explained by the exhaustion of body fat, by the discontinuance of alcohol consumption, and by the resumption of a normal or a high carbohydrate diet.

# *The Journal of Laboratory and Clinical Medicine*

---

VOL. 24

NOVEMBER, 1938

No. 2

---

## *CLINICAL AND EXPERIMENTAL*

---

### *PLETHYSMOGRAPHIC STUDIES WITH SPECIAL REFERENCE TO WAVES OF RESPIRATION\**

---

S. J. MARTIN, M.D., F. S. MARCELLUS, AND P. SYKOWSKI, B.A., ALBANY, N. Y.

---

THE effect of respiration on blood pressure in the larger arteries was investigated many years ago by Lewis<sup>1</sup> and Erlanger and Festerling.<sup>2</sup> Further studies of vascular phenomena have been facilitated greatly by the use of the plethysmograph, and recently emphasis has been placed upon the peripheral portion of the vascular tree. Johnson<sup>3</sup> described a sensitive glass tube finger plethysmograph in which the volume changes were recorded by photographing the movements of a drop of colored alcohol in a horizontal calibrated tube attached to the plethysmograph. From these photographic records it was possible to estimate a 0.002 c.c. change in volume. In subsequent studies<sup>4,5</sup> reference was made to superimposed waves associated with respiration which were believed to be due to (a) periodic vasomotor changes, (b) changes in cardiac output, or (c) movement of the animal. Bolton, Carmichael, and Stürup<sup>6</sup> used an optical system of flat mirrors to record photographically the vascular changes in the finger, but gave no special significance to waves synchronous with respiratory movements. Turner<sup>7</sup> described a new sensitive apparatus consisting of a pulse recorder, optical capsule, and photokymograph to study vascular phenomena in the terminal phalangeal region. The appearance of waves of respiration was not mentioned.

Using a new and sensitive apparatus in the study of peripheral vascular phenomena, waves of respiration were recorded along with pulse and volume changes. An attempt in this investigation was made to calibrate this apparatus

---

\*From the Department of Physiology and Pharmacology, Albany Medical College, Albany, N. Y., and the General Engineering Laboratory, General Electric Co., Schenectady, N. Y.

Received for publication, March 8, 1938.

and also to determine the underlying factors responsible for the appearance of the respiratory waves. A preliminary report of this study has appeared.<sup>8</sup>

#### PROCEDURE AND RESULTS

The apparatus used for this study was constructed by the General Electric Co., Schenectady, N. Y. It is a photoelectric recorder which combines all the advantages of direct acting recorders with those of the most sensitive instruments. Plethysmographic changes are transmitted by simple rubber tubing to the basic element or tambour mounted on the apparatus. Any motion of the tambour in turn moves a small mirror which, when in equilibrium, casts a beam of light exactly between two photoelectric cells. With the aid of an optical system of mirrors, imbalance of the central beam activates the right or left photoelectric cell. This procedure accordingly moves a siphon type recording pen which follows an indicating element and records on a continuous strip chart (Fig. 1). The apparatus is simple to manipulate and affords one a direct record and without recourse to photographic procedures. The records can be readily reprinted.

1. *Calibration.*—Preliminary studies in peripheral vascular phenomena revealed the extreme sensitivity of this new type of recording device. An attempt was, therefore, made to calibrate it. A circular pyrex glass chamber (9.0 by 2.5 cm.) was constructed with three outlets, the center one of which was attached to a microcapillary pipette graduated to 0.001 c.c. One outlet was connected to the recording device and the other served for introduction of a colored fluid. A 1 c.c. tuberculin syringe was attached to the pipette to facilitate withdrawal of fluid from the glass chamber. Pinch clamps were placed at one outlet and between the pipette and chamber. Beginning with the colored fluid in the pipette at zero, increments of 0.001 c.c. were introduced into the chamber and the magnitude of deflection of the pointer noted. This procedure was continued until a maximal deflection was noted and then the deflections were measured upon withdrawing 0.001 c.c. increments.

It was found that the magnitude and accuracy of the deflections, when the tambour of the apparatus was fixed at zero, depended upon the internal diameter and not upon the length of the tube connecting the chamber and photoelectric recorder. Rubber tubes of nine different lengths and of four different diameters were used. The most satisfactory tube for animal work had an internal diameter of 0.15 cm. and an outside diameter of 0.4 cm. The length could be variable up to 175 cm. With such a tube, the siphon pen of the apparatus showed a deflection to either side of the midline of 48 mm. representing an increase in volume in the chamber of 0.16 c.c. Therefore, 1 mm. deflection was equivalent to a change of 0.003 c.c. in volume. Changes of 0.001 c.c. could be detected, but only the 0.003 c.c. increment could be accepted as accurate.

Although this apparatus requires little technical knowledge to manipulate it, it is exceptionally sensitive and accurate. The only significant precaution in its use is to avoid extraneous vibrations or undue movement of the animal. The calibration, in particular, has served to form a basis for quantitative study of peripheral vascular phenomena in man.

2. *Factors Concerned With Waves of Respiration in Plethysmographic Studies*—In determining the role played by nervous and mechanical factors in the appearance of respiratory waves, plethysmographic records were made of the fingers and toes of 3 men and 3 monkeys, and of the hind paw of 3 rabbits,

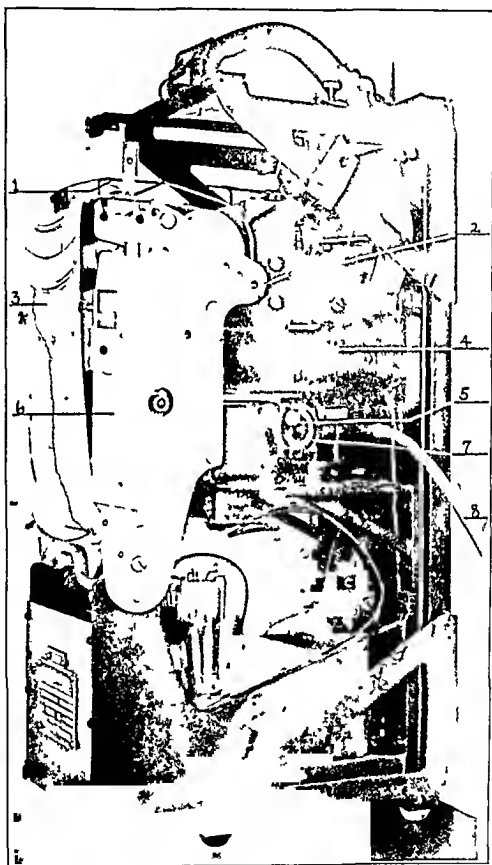


Fig 1—The photoelectric recorder used in plethysmographic studies of digits of man and of the paw of animals. 1, siphon pen, 2, indicating element, 3, strip chart, 4, photoelectric cell, 5, mirror, 6, chart carriage, 7, basic element or tambour, 8, rubber tube to plethysmograph (Through the courtesy of the General Electric Co. Schenectady, N. Y.)

5 rats, 5 guinea pigs, 5 cats, and 22 dogs. The plethysmographs were made of pyrex glass tubing, one end of which was connected to the photoelectric recorder and the other end snugly attached with a synthetic nonelastic rubber

dam and a layer of vaseline at the second interphalangeal region, generally of the finger, or at the first interphalangeal region of the toe. In animals, the plethysmograph was attached 1 to 5 cm. proximal to the pad of the hind paw. Air leakage was eliminated before experimentation.

In addition to the pulse and volume changes, waves synchronous with respiration could be noted in all observations made, except in the case of guinea pigs and rats and in a number of instances in the toes of man and monkey

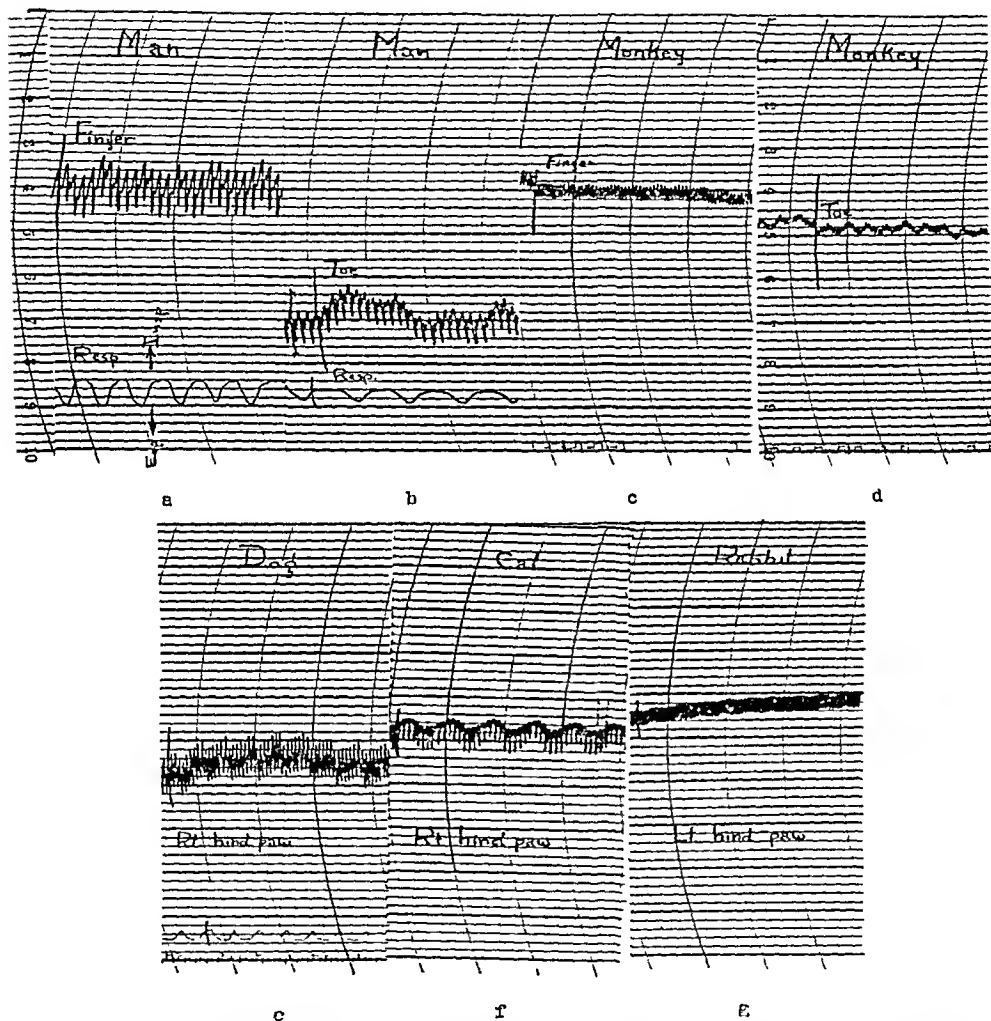


Fig. 2.—Plethysmographic records taken on a continuous strip by the photoelectric recorder of the digits of man and monkey and of the hind paw of animals. Note the pulse rate and waves synchronous with respiration. The bottom tracing in a, b, and c represents respiratory chest movements. Decrease in volume of the digits is noted by movement of the pointer toward the zero mark; increase in volume or vasodilation, by movement toward the ten mark. Line 5 represents the normal or initial volume. The vertical lines indicate ten-second intervals. Waves synchronous with respiration may be seen in records of all animals.

(Fig. 2). When these respiratory waves were present, a definite correlation could be established between plethysmographic volume and phases of respiration. Decrease in volume followed inspiration and increase in volume generally was seen after expiration. It was significant to note that the magnitude of the

respiratory waves in the plethysmograph tracing was directly proportional in all cases to the increase in plethysmographic volume. This correlation was greatly magnified in cats and dogs anesthetized and receiving endotracheal artificial respiration. In these animals if the inspiratory phase was purposely prolonged, a decrease in the plethysmographic volume of the paw was noted and the respiratory waves disappeared. However, in the sustained phase of expiration, the result was variable in that either vasoconstriction or slight or marked vasodilation occurred (Fig 3).

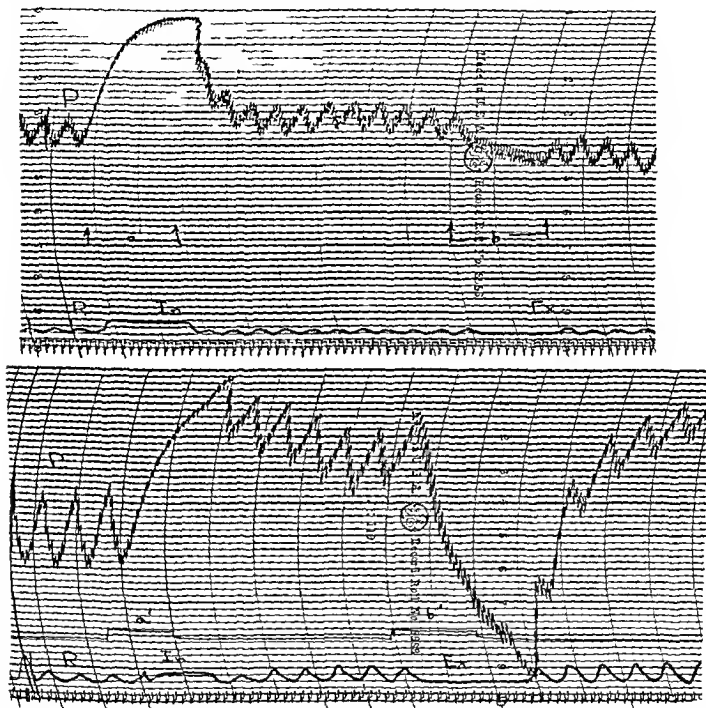


Fig 3—Plethysmographic records (P) of the hind paw of dogs receiving endotracheal artificial respiration (R). The upper record shows vasoconstriction during sustained inspiration (In). Slight vasodilation is seen during expiration (Ex). In the lower record marked vasodilation is noted during the expiratory phase (Ex).

*a Mechanical Factor*—The importance of the mechanical factor responsible for the appearance of the waves of respiration was revealed in the following experiments. The glass plethysmograph was attached to the right or left hind paw of 5 cats and 9 dogs and under nembutal anesthesia, the corresponding femoral nerve and femoral and iliac vessels were exposed. This surgical procedure resulted in a transient reflex increase in heart rate, a vasoconstriction



of the vessels of the paw followed by a vasodilation. The changes in the amplitude of the respiratory waves were secondary to the degree of vasoconstriction noted.

No change was noted in character of the respiratory waves, except a slight increase in magnitude due to the vasodilation in the paw, after temporary and light clamping of the femoral or common iliac veins. When the femoral artery

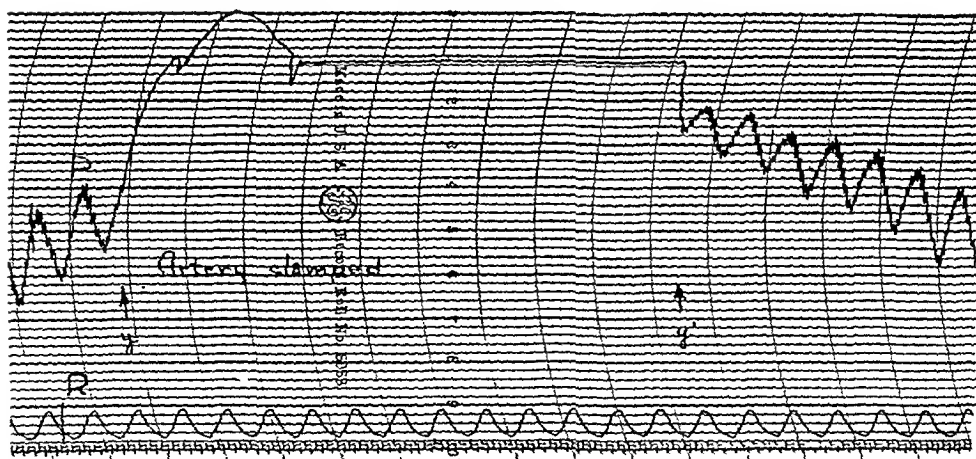
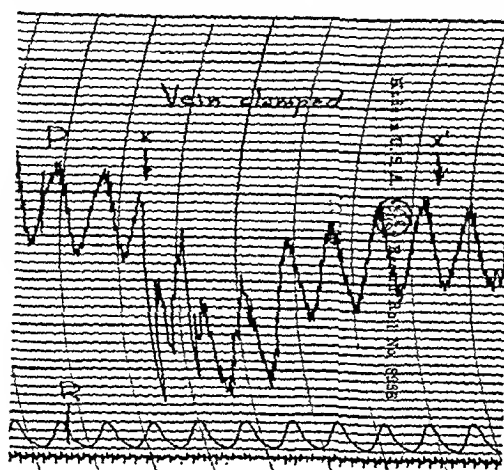


Fig. 4.—Plethysmographic records (P) of the hind paw of a dog receiving endotracheal respiration (R). Upper record: marked vasodilation upon temporary clamping ( $x-x'$ ) of the femoral vein with increase in amplitude of respiratory waves. Lower record: no respiratory waves during the sudden decrease in blood volume produced by temporary clamping ( $y-y'$ ) of the femoral artery. Return of the waves of respiration to normal height can be noted in both records upon release of clamps.

was occluded with fingers, of course, the pulse disappeared along with the respiratory waves despite the intact innervation (Fig. 4). In 3 of the dogs, the vessels were painted with 70 per cent alcohol solution and in 3 others the connective tissue coat for 3 to 4 cm. was stripped in order to eliminate the influence of the sympathetic fibers. No change was noted as compared to the

above results when the different vessels were clamped. It would seem, therefore, that the appearance of the waves of respiration was due to mechanical causes rather than to a nervous influence.

In another series of 5 dogs, plethysmographic records were taken of the hind paw after the leg was severed from the body at the inguinal region, except for the femoral artery and vein. Temporary clamping of the vein again

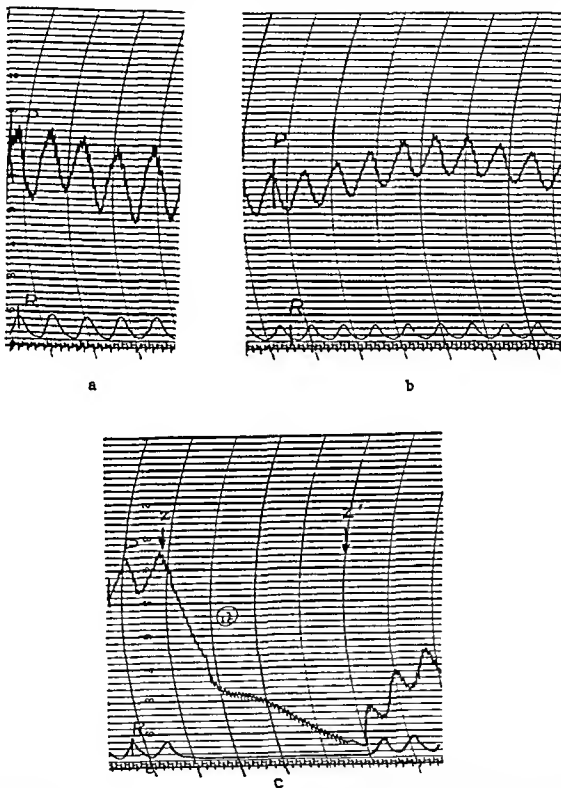


Fig 5—*a* 1 artificial respiratory ends of the femoral artery and vein. Note the decrease in blood

ing of hind paw of a dog under endotracheal was inserted between proximal and distal cut severed from the body except for the vessels *r*<sub>1</sub> waves *c* Absence of respiratory waves and porary cessation of respiration (*s-s'*)

did not influence the appearance of the respiratory waves, while occlusion of the artery did. To rule out all possible nervous impulses coming from the body, a glass cannula was inserted between the proximal and distal cut ends of the femoral artery in these dogs. The magnitude of the respiratory waves

was diminished about 33 per cent, but the waves were nevertheless clearly evident (Fig. 5a, b). This decrease in size may have been due (1) to removal of the nervous factor, or (2) to clot formation in the cannula with resultant diminished blood flow to the paw. The respiratory waves in these cannulated preparations were magnified by increased thoracic excursions and promptly disappeared when the artery was pinched off, or when the tracheal artificial respiration was temporarily stopped (Fig. 5c). In 3 of these animals receiving artificial respiration, the diaphragm was resected and the abdomen closed. Such a procedure did not eliminate the appearance of the respiratory waves. However, when the chest and abdomen were subsequently opened, the respiratory waves immediately disappeared despite the fact that the heart was beating and the lungs were expanding under artificial respiration. The remaining 2 dogs were sacrificed by an intravenous injection of pentobarbital, and the artificial respiration was continued after the cessation of the heart beat.

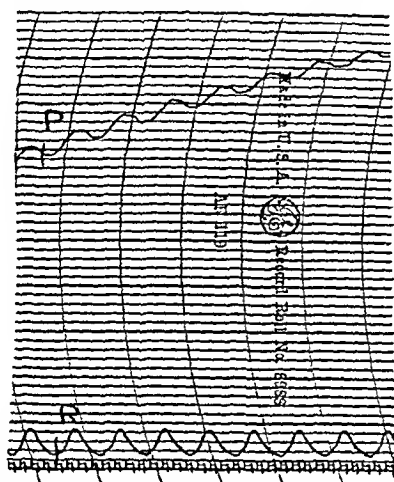


Fig. 6.—Plethysmographic record of hind paw of a recently dead and pulseless dog still receiving artificial respiration. Waves synchronous with respiration are still present.

Plethysmographic records of the paw in these recently dead and pulseless dogs still receiving artificial respiration revealed the presence of respiratory waves, although to a markedly diminished extent (Fig. 6).

It was concluded, therefore, that the appearance of superimposed waves of respiration in plethysmographic records of the paw are primarily caused by the mechanical factor due to the movement of the thoracic cage upon the blood flow. The magnitude of these waves appears dependent upon the degree of arterial relaxation in the paw.

b. *Nervous Factor*.—Because of the possibility of a nervous influence in the decrease of the amplitude of the respiratory waves in the cannula experiments, referred to above, it was considered advisable to investigate this factor more thoroughly. Accordingly, plethysmographic records of the hind paw of 8 additional dogs were studied in the following experiments. Under nembutal anesthesia, the femoral and iliac vessels and nerves were exposed, and the nerves cut. No effect was noted on the waves of respiration. In 4 of these animals, a

transection of the spinal cord at the twelfth thoracic and first lumbar region was performed. Similarly, the respiratory waves remained unchanged.

Mild to moderate faradic stimulation was applied to the central and peripheral cut ends of the femoral and sciatic nerves and the peripheral region of the transected cord. In all instances the magnitude of these waves was dependent upon primary reflex changes in the size of the vessels. Thus, with each vasoconstriction in the paw the waves decreased in size, and conversely, upon vasodilation they increased. In no case were they absent. When endotracheal artificial respiration was temporarily stopped, faradic stimulation to the cut nerves or spinal cord did not cause the waves to reappear. Thus, it may be concluded that the somatic or spinal nerves exert no influence on the appearance of the waves of respiration.

In 3 of the 4 dogs with a cord transection and section of the femoral and sciatic nerves, a moderate faradic stimulus was applied to the exposed femoral artery for the purpose of stimulating the sympathetic nerves. The waves of respiration detected in the arteriolar bed of the paw did not disappear, although their magnitude was decreased due to the slight vasoconstriction produced. Thus, the sympathetic innervation is not primarily concerned with the appearance of the respiratory waves.

#### DISCUSSION

The influence of thoracic movements on blood pressure is well known and can be readily noted in recording the pressure of aortic, carotid, and femoral arteries and in plethysmographic studies of the spleen and kidney. In regions where arterioles predominate, such as the digits of man and the paw of animals, little attention has been paid to waves synchronous with respiration. These waves have been detected with the use of the photoelectric recorder in the peripheral portion of the vascular tree. Our results indicate that they are mechanical in origin due to the increased pressure exerted on the thoracic and abdominal arterial blood by respiratory movements. The use of the sensitive photoelectric recorder has enabled us to detect such minute increases in pressure of the blood in the arteriolar bed of the paw. That they may be due to changes in cardiac output has not been entirely ruled out. Their prompt disappearance when artificial respiration is temporarily stopped or when the chest or abdomen is opened may depend in part upon decreased cardiac filling.

Our data also show that no direct nervous factor is involved in the transmission of the respiratory pulsations. However, upon inspiration there is a peripheral vasoconstriction which according to Goetz<sup>9</sup> and Bolton, Carmichael, and Sturup,<sup>6</sup> is of reflex origin. These vasomotor impulses to the arterioles of the paw secondarily influence the magnitude of the respiratory waves. Thus with vasoconstriction, the waves of respiration decrease in amplitude. Extraneous factors, such as movement of the animal or vibrations of the animal board, have been ruled out by the use of long rubber tubing connecting the plethysmograph and the photoelectric recorder placed on different tables.

This investigation does not confirm the suggestion made by Johnson<sup>4</sup> that the appearance of waves of respiration in plethysmographic studies of arteriolar regions is due to vasomotor action or to body movements of the animal.

Since the magnitude of the waves of respiration depends upon the patency of the arteriolar bed of the vascular system, their detection may be of clinical value. Thus, by determining the amplitude of the respiratory waves in response to the use of suitable vasomotor stimuli, one can differentiate between mechanical and nervous factors in the etiology of peripheral vascular disease. Such a procedure could be of prognostic as well as of diagnostic importance. Further, the relative stability of the vasomotor system and deviations from the normal response to certain arbitrary stimuli may be determined by noting the changes in the size and character of the respiratory waves in plethysmographic studies of digits.

The use of our plethysmograph and photoelectric recorder, aside from the detection of the waves of respiration, has served to record pulse rate and volume changes in digits. This procedure can be utilized in the study of peripheral vascular responses to vasomotor drugs, anesthetics, and to varying degrees of surgical trauma. The apparatus possibly may be used to show pressure and volume changes in arteries, uteri, or intestinal loops.

#### SUMMARY

A sensitive photoelectric recorder was calibrated and used in plethysmographic studies of digits of man and of the paw of animals. The apparatus was easy to manipulate and afforded one a direct record without recourse to photographic procedures. In addition to pulse rate and volume changes, waves synchronous with respiration were recorded. These waves were found to be mechanical in origin and essentially due to increased pressure with each respiration of the thoracic and abdominal blood, a pressure transmitted to the paw. Nervous factors have been ruled out. The magnitude of the respiratory waves, however, depends upon the degree of arteriolar relaxation. It is suggested that the appearance and character of these waves may be of importance in the study of peripheral vascular phenomena in man.

#### REFERENCES

1. Lewis, T.: Studies of the Relationship Between Respiration and Blood Pressure, *J. Physiol.* 37: 213, 1908.
2. Erlanger, J., and Festerling, E. G.: Respiratory Waves of Blood Pressure, With an Investigation of a Method for Making Continuous Blood Pressure Records in Man, *J. Exper. Med.* 15: 370, 1912.
3. Johnson, C. A.: The Effect of Amyl Nitrite Upon the Finger Volume, *J. Lab. & Clin. Med.* 17: 59, 1931.
4. Johnson, C. A.: Studies on Peripheral Vascular Phenomena. I. A New Device for the Study of Peripheral Vascular Phenomena in Health and Disease, *Surg., Gynec. & Obst.* 55: 731, 1932.
5. Johnson, C. A., Scupham, G. W., and Gilbert, N. C.: Studies on Peripheral Vascular Phenomena. II. Observations on Peripheral Circulatory Changes Following Unilateral Cervical Ganglionectomy and Ramisection, *Surg., Gynec. & Obst.* 55: 737, 1932.
6. Bolton, B., Carmichael, E. A., and Stürup, G.: Vasoconstriction Following Deep Inspiration, *J. Physiol.* 86: 83, 1936.
7. Turner, R. H.: Studies in the Physiology of Blood Vessels in Man. Apparatus and Methods. I. A Sensitive Plethysmograph for a Portion of the Finger, *J. Clin. Investigation* 16: 777, 1937.
8. Martin, S. J., and Marcellus, F. S.: Plethysmographic Studies With Special Reference to Waves of Respiration, *Am. J. Physiol.* 119: 373, 1937.
9. Goetz, R. H.: Der Fingerplethysmograph als Mittel zur Untersuchung der Regulationsmechanismen in peripheren Gefäßgebieten, *Pflüger's Arch. f. d. ges. Physiol.* 235: 271, 1935.

## NEUTROPENIA FOLLOWING SULFANILAMIDE\*

### REPORT OF A CASE

HERBERT W. JONES, M.D., AND C. PHILIP MULLER, M.D. CHICAGO, ILL.

IT WAS to be expected that the current increase in the use of sulfanilamide would give rise to occasional instances of toxicity. The occurrence of fever and skin rashes as toxic manifestations has been noted<sup>1</sup> and three cases of acute hemolytic anemia have been reported by Harvey and Janeway,<sup>2</sup> another by Kohn,<sup>3</sup> and one case of severe and two of moderate anemia by Jennings and Southwell Sander.<sup>4</sup> In addition to these six instances of malignant neutropenia have been recorded in this country and England. Only one of the four recovered.

### LITERATURE

The first report of agranulocytosis attributable to sulfanilamide is by Plumer.<sup>5</sup> A woman of 54, suffering from subacute endocarditis (*streptococcus viridans*) of about five months' duration was given 1 gm. of sulfanilamide a day for four days, and 13 gm. for thirty one days, when it was discontinued because of nausea and vomiting. Three days thereafter the white count had fallen to 400, with no neutrophiles, it rose the next day to 1,600, but extensive gangrenous infection of the mouth and pharynx had already developed and death occurred on that day.

Young<sup>6</sup> reports agranulocytosis with fatal outcome in a man of 53 years, suffering from acute rheumatism, who had taken 3 gm. of sulfanilamide for eighteen days. Skin rashes (not described) are mentioned as occurring on four days, the first of which antedated the beginning of sulfanilamide administration. Model's<sup>7</sup> case was a man of 20 suffering from a recurrence of rheumatic fever. Three grams of sulfanilamide were given for eighteen days and then discontinued. Two days later the leucocyte count was found to be 600. In spite of blood transfusion and pentnucleotide, death occurred the following day. Jennings and Southwell Sander<sup>4</sup> observed agranulocytosis in a 39 year old woman who suffered from ulcerative colitis. Sulfanilamide, 45 gm. per day, had been given for three weeks and discontinued because her symptoms had disappeared. Two days later evidence of agranulocytosis began and increased in severity for four days, when the total white blood count was less than 400, none of which were polymorphonuclear cells. The administration of pentnucleotide was followed immediately by a rapid rise in the polymorphonuclear count and general improvement and recovery.

\*From the Department of Medicine and the Kuppenheimer Foundation of the University of Chicago.

Received for publication March 14 1938

Quite recently two additional cases have been added. The patient of Schwartz, Garvin, and Koletsky<sup>8</sup> was a man of 32, treated for a penile ulcer with sulfanilamide over a period of twenty-one days, during which time he took a total of 56.6 gm. The typical symptoms of agranulocytosis developed and resulted in death. Berg and Holtzman<sup>9</sup> report a fatal attack of agranulocytosis in a man of 22 years, who took 38 gm. of sulfanilamide in eleven days for acute gonorrhea.

The gravity of neutropenia as a toxic manifestation of a drug so widely used as sulfanilamide prompts the present authors to add another case report to the literature, for it is only by the gradual accumulation of such data that the administration of this valuable drug can be safeguarded.

#### CASE REPORT

The patient, N, a native-born white man, 26 years old, an unemployed salesman, was admitted to the University of Chicago Clinics, November 5, 1937, suffering from acute gonococci anterior urethritis (his first attack), which had begun the day before, five days after

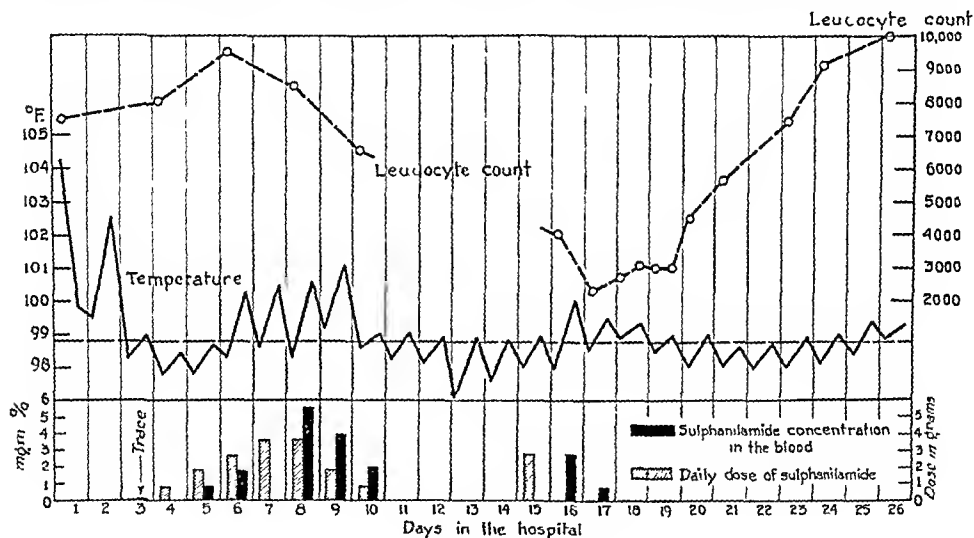


Fig. 1.

exposure. He was treated in the Urological Out-Patient Department, where 0.6 gm. sulfanilamide was ordered to be taken four times a day. At subsequent visits on November 8 and 11 his discharge was found to contain gonococci, and sulfanilamide therapy was continued. On November 15 he reported that he had been having headache, backache, and fever for three days, during which period he had taken very little food or fluid. His urethral discharge had ceased. The urine was clear, containing only an occasional shred and occasional white blood cell. His temperature was 104.4° F.

He was transferred to the medical service and admitted to the Albert Merritt Billings Hospital. Chief complaints were headache, backache, and pains in his legs, especially the knees. Positive findings on physical examination were temperature, 104.2° F., dryness of the tongue and skin, distinct odor of acetone in the breath (although none could be demonstrated in the urine), moderate injection of the pharynx, and a soft blowing systolic murmur heard over the third interspace, just to the left of the sternum.

The temperature curve and leucocyte counts are given in the accompanying chart as well as the sulfanilamide content of the blood as determined by Marshall's method.<sup>10</sup> Blood cultures made on each of the first three days remained sterile, and cultures of the nose,

throat, and stools developed only the customary inhabitants of the upper respiratory and intestinal tracts. Urinalyses were uniformly negative, except for leucocytes and occasional red cells. Blood Wassermann and Kahn were negative.

As certain of the toxic rashes described seemed to have been brought out by sunlight, one treatment with ultraviolet light was given over the left arm. Only a mild erythema resulted.

#### TOTAL AND DIFFERENT LEUCOCYTE COUNTS\*

DAY	TOTAL	POLYMORPHONUCLEAR NEUTROPHILES	BASOPHILS	LYMPHOCYTES	MONOCYTES
16	4,100	25	5	64	1
17	2,300	5	3	77	12
18	2,600	9		52	31
19	3,100	5		64	21
20	3,000	15	2	59	21
21	4,500	25	1	57	13
22	5,600	26	1	49	11
23	7,400	11	3	50	5
24	9,100	77	1	30	10
26	10,000	64	1	32	3

\*For most of these counts the authors are indebted to Miss Alice Plain

The patient's symptoms subsided with his fever, and the heart sounds became clear, but on the fourth day the urethral discharge returned and contained numerous gonococci. Sulfanilamide therapy was cautiously resumed, beginning with 0.9 gm and increasing by that amount each day until a daily dose of 3.6 gm was reached. His temperature rose again, and on the second day of maximum dosage the patient complained of mild dull headache, chilliness, general malaise, and the feeling "that he was coming down with a cold." The drug was discontinued and his fever and symptoms disappeared. During this period his leucocyte count remained normal.

The urethral discharge, which had lessened under full doses of sulfanilamide, once more increased, and on the fifteenth day 2.7 gm. of the drug were administered. The next morning the urethral discharge, which had been examined daily, was found to contain few healthy looking polymorphonuclear cells, and the blood count showed only 4,100 leucocytes, of which 25 per cent were polymorphonuclears. Later that day the patient complained again of headache and chilliness, general malaise, and the feeling "that he was coming down with a cold." On the following day the leucocyte count was 2,300, neutrophils only 5 per cent. Yellow bone marrow concentrated,\* 80 drops a day, was given and continued for six days. The leucocyte count began to rise the next day (the eighteenth), and continued to do so. His symptoms improved and disappeared entirely on the nineteenth day. By the twentieth day the urethral discharge, which had been scant and serous since the sixteenth day, became more profuse and purulent. At no time were gonococci absent. The patient was discharged from the hospital December 18, 1937, and transferred back to the Urological Clinic for ambulatory treatment of his urethritis, from which he had not yet recovered.

#### COMMENT

Although some doubt was entertained at the time of this patient's admission to the hospital that he was suffering from a febrile reaction to sulfanilamide, subsequent events seem to have established this diagnosis with certainty. The sulfanilamide determination on the blood drawn the first day was unreliable, so its content at the height of his symptoms is unknown. But by the third day, when his blood showed only a trace of sulfanilamide, the patient was symptom free; and after the drug had been increased again to its former dosage, the fever and some of his symptoms once more returned.

\*Supplied by Armour and Company, Chicago



During this second period of intoxication, when he was suffering from mild headache, general malaise, and the feeling "that he was coming down with a cold," the concentration of sulfanilamide (free plus "combined") in the blood was only 5.6 mg. per cent, less than one might expect from the dose he had taken, probably because of the high level at which fluid intake and output was maintained. Marshall and his co-workers<sup>11</sup> have emphasized the rapidity of sulfanilamide excretion by the kidneys.

The same symptoms recurred for the third time when the blood contained only 2.8 mg. per cent of sulfanilamide, and this time were accompanied by severe neutropenia. It seems highly probable, therefore, that this patient was becoming increasingly susceptible to the drug.

It must be borne in mind that this patient had taken 2.4 gm. of sulfanilamide a day for ten days before his admission to the hospital. In this connection it will be recalled that in the reported cases of agranulocytosis cited in this paper, the drug had been administered over periods varying from eleven to thirty-five days.

One is led to the conclusion that vulnerability to the toxic action of sulfanilamide developed about the beginning of the second week of its administration and increased during the period of observation here reported. Accumulation of the drug in the body seems to have been ruled out by the estimations of its concentration in the blood. Hageman and Blake,<sup>1</sup> who observed fever, sometimes accompanied by skin rash on the tenth to twelfth day, were unsuccessful in their effort to prove it an allergic phenomenon.

#### SUMMARY

A patient who had taken sulfanilamide for eight days developed fever and generalized aching pains. Two attempts to resume therapy were followed by a return of symptoms, the last time accompanied by severe neutropenia. Determinations of its concentration in the blood indicate increasing susceptibility to the drug.

#### REFERENCES

1. Hageman, P. O., and Blake, Francis G.: A Specific Febrile Reaction to Sulfanilamide, *J. A. M. A.* 109: 642, 1937.  
Schwentker, Francis F., and Gelman, Sidney: Sulfanilamide Rash, *Bull. Johns Hopkins Hosp.* 61: 136, 1937.  
See also six papers in *J. A. M. A.* 109: 999, 1005, 1007, 1008, 1009, 1011, 1937.
2. Harvey, A. M., and Janeway, C. A.: The Development of Acute Hemolytic Anemia, *J. A. M. A.* 109: 12, 1937.
3. Kohn, S. E.: Acute Hemolytic Anemia During Treatment With Sulfanilamide, *J. A. M. A.* 109: 1005, 1937.
4. Jennings, G. H., and Southwell-Sander, G.: Anemia and Agranulocytosis During Sulfanilamide Therapy, *Lancet* 2: 898, 1937.
5. Plumer, Herbert E.: Letter to New England *J. Med.* 216: 711, 1937.
6. Young, C. J.: Agranulocytosis and Para-aminobenzenesulphonamide, *Brit. M. J.* 2: 105, 1937.
7. Model, Alfred: Agranulocytosis and Para-aminobenzenesulphonamide, *Brit. M. J.* 2: 295, 1937.
8. Schwartz, W. F., Garvin, Curtis F., and Koletsky, Simon: Fatal Granulocytopenia From Sulfanilamide, *J. A. M. A.* 110: 368, 1938.
9. Berg, Samuel, and Holtzman, Michael: Fatal Granulocytopenia From Sulfanilamide, *J. A. M. A.* 110: 370, 1938.
10. Marshall, E. K.: Determination of Sulfanilamide in Blood and Urine, *Proc. Soc. Exper. Biol. & Med.* 36: 422, 1937.
11. Marshall, E. K., Emerson, Kendall, Jr., and Cutting, W. C.: The Renal Excretion of Sulfanilamide, *J. Pharmacol. & Exper. Therap.* 61: 191, 1937.

## A NOTE ON THE DIAGNOSIS OF HYPERTENSIVE CARDIOVASCULAR DISEASE WITHOUT HYPERTENSION\*

EDWARD H. SCHWAB, M.D., AND DOROTHY L. CUTH, M.D. GALVESTON, TEX.

THE middle aged cardiopath who presents moderate to marked cardiac enlargement, an absence of significant valvular lesions, and normal or low blood pressure, with or without signs of heart failure constitutes an interesting diagnostic problem from an etiologic viewpoint. According to present day cardiology teaching, the diagnoses of "chronic myocarditis" and "chronic nonvalvular disease of the heart," usually given to cases of this type, are no longer acceptable.

Coronary artery disease forms the background for this clinical syndrome in certain instances, although in these cardiac hypertrophy of great degree is an unusual finding. Other inciting factors in a small number of cases are chronic adhesive mediastinopericarditis, syphilitic coronary ostial disease, and latent or masked hyperthyroidism. The large majority of such cases, however, as has been emphasized by O'Hare and his associates,<sup>1</sup> are probably instances of hypertensive cardiovascular disease in which the blood pressure has fallen to a normal, or in some cases a subnormal, level.

The variability of the blood pressure in essential hypertension is well known. Ajman<sup>2</sup> reported the finding of normal blood pressures in 56 per cent of 76 cases of established essential hypertension, some of which exhibited widespread vascular changes. Such spontaneous remissions in blood pressure are as a rule transitory and of relatively short duration. With the advent of heart failure of the congestive type in hypertensive cases marked reductions in blood pressure to normal or subnormal levels are not uncommonly encountered and are frequently persistent. In some instances, with the restoration of cardiac compensation, the blood pressure returns to its previous high level. In establishing an etiologic diagnosis in these cases of hypertensive cardiovascular disease without hypertension seen for the first time, O'Hare<sup>1</sup> has emphasized the importance of an antecedent history of hypertension, and the presence of retinal arteriosclerosis. The former, however, while of great importance, is frequently lacking. Additional confirmatory findings would appear to be the presence of a diffuse fibrous type of peripheral arteriosclerosis, arteriolar sclerosis determined by muscle biopsy and, perhaps, evidence of impairment of renal function.

The important contributions of Hines and Brown<sup>3</sup> concerning the blood pressure response to their standard vasomotor stimulus, the cold pressor test, have revealed that 98 per cent of patients with essential hypertension give abnormal maximal responses. From these findings they conclude that patients

\*From the Department of Practice of Medicine, Medical Branch, University of Texas and the Cardiac Clinic of the John Sealy Hospital, Galveston.  
Received for publication March 14, 1938.

with essential hypertension possess hyperreactive vasomotor mechanism. These observers further referred to "a group of healthy subjects in the later decades of life who had hyperreactions and changes in the retinal arterioles which were indicative of essential hypertension, but who had normal or subnormal levels of (resting) blood pressure." It occurred to us that in suspected hypertensive cardiopaths presenting normal or low blood pressures, such hypersensitivity of the vasomotor apparatus to stimulation might perhaps be retained, even though the "resting" blood pressures were no longer elevated. If so, the procuring of a maximal, or "hypertensive type" of response to the standard stimulus of such a case would furnish important confirmatory evidence toward establishing an accurate etiologic diagnosis. During the prosecution of our studies on the cold pressor test we have made certain observations which tend to show that in the majority of instances such appears to be the case.

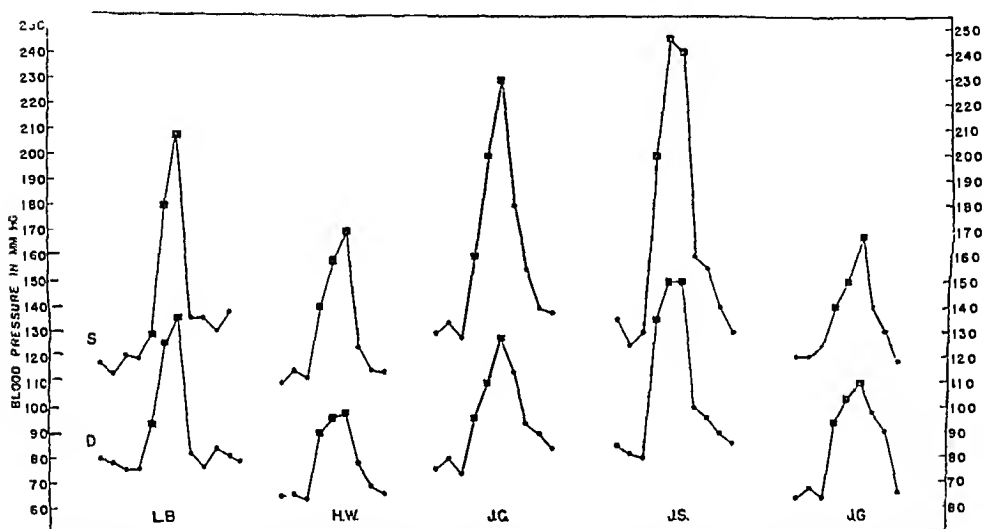


Chart 1—Blood pressure response to the cold pressor test in five subjects with hypertensive cardiovascular disease with normal blood pressures. An excessive or "hypertensive" type of response was obtained in each instance. The squares represent readings taken during the application of the stimulus.

Seven subjects with known hypertensive cardiovascular disease were encountered in whom the blood pressures had fallen to normal or subnormal levels. These patients had been under observation for from two to ten years, during which time there had been persistent and marked elevation of both the systolic and diastolic pressures. In addition, all showed cardiac enlargement as well as retinal and peripheral arteriosclerosis. All were males, their ages ranging from 35 to 55 years. The apparent cause of the blood pressure reduction, in five cases, was congestive heart failure; in one case, coronary artery closure with cardiac infarction; and in the other case, a streptococcus throat infection.

We subjected all seven of these patients to the cold pressor test. The vasomotor stimulus employed was that of Hines and Brown,<sup>3</sup> with certain minor modifications.<sup>4</sup> Blood pressure readings were taken at intervals of thirty, ninety, and one hundred and fifty seconds during the period of stimulus

(cold) application, instead of the two readings at thirty and sixty seconds as in the original method

Five of the seven subjects gave a decided maximal or "hypertensive type" response, according to our criteria (Chart 1). Of the remaining two, one gave an essentially normal response, and the other a borderline type of reaction. In the latter instance it was felt that a primary nephritis might possibly have been the etiologic factor of the hyperpiesia.

From these results the conclusion seems justified that in many patients with hypertensive heart disease in whom the blood pressure has fallen to a normal or subnormal level, the hyperirritability of the vasomotor apparatus is retained and vasomotor stimulation will evoke an exaggerated blood pressure rise similar to that observed in the usual hypertensive subject *with* hypertension. It would seem, therefore, that the exhibition of such a response in a cardiac patient of the type described would provide a valid reason for believing that a pre-existing hypertension was the important etiologic factor in the cardiac pathology presented.

#### SUMMARY

Seven patients with established hypertensive cardiovascular disease whose blood pressure had fallen to normal or subnormal levels were subjected to the cold pressor test and the blood pressure response noted. In five instances the typical hypertensive type of response was obtained. This fact suggests that the application of such a procedure to patients of this type would materially aid in the clarification of the etiologic cardiac diagnosis.

#### REFERENCES

- 1 O'Hare, J. P., Culhoun, A. W., and Altnow, H. O. Etiology of Chronic Myocarditis, *J. A. M. A.* 90: 1436, 1928.
- 2 Ayman, David. Normal Blood Pressure in Essential Hypertension, *J. A. M. A.* 94: 1214, 1930.
- 3 Hines, E. A., Jr., and Brown, G. E. The Cold Pressor Test for Measuring the Reactibility of the Blood Pressure. Data Concerning 571 Normal and Hypertensive Subjects, *Am. Heart J.* 11: 1, 1936.
- 4 Schwab, E. H., Curb, D., Matthews, J. T., and Schulze, V. E. Blood Pressure Response to a Standard Stimulus in the White and Negro Races, *Proc. Soc. Exper. Biol. & Med.* 32: 583, 1935.

# ON THE NATURE OF THE ANTIPERNICIOUS ANEMIA PRINCIPLE IV\*

## SEARCH FOR NITROGENOUS BASES ISOLATION OF CHOLINE

HENRY R. JACOBS, M.D., CHICAGO. ILL.

THE response of a patient with pernicious anemia to a mixture of potato juice and tyrosine was described in an earlier paper.<sup>1</sup> The "red substance" of Raper was suspected to be concerned with the beneficial effect observed. Further clinical tests with (1) dihydroxyphenylalanine, (2) dihydroxyphenylalanine oxidized with peroxide and traces of iron salts, and (3) dihydroxyphenylalanine oxidized by oxygen in alkaline solution have shown, through uniformly negative results, that this idea was probably erroneous.

Because, however, a reticulocyte response had been observed after giving potato juice and tyrosine, it seemed justifiable to examine potent preparations for the presence of some of the simpler nitrogenous bases known to occur in potatoes and other plants.<sup>2</sup> The group of bases including choline and the betaines (betaine, trigonelline, stachydrine, etc.) was selected for study because they, like the active principle in liver extract, form precipitates with phosphotungstic acid.<sup>3</sup>

A preliminary test of liver extract with potassium periodide (Stanek) and with potassium bismuth iodide (Kraut) established the presence of much material precipitable with these reagents. If liver extract is first treated with lead acetate and the excess of lead removed, these "alkaloidal reagents" often yield crystalline precipitates. Although these precipitates are formed readily, they are not in themselves suitable for the positive identification of specific substances. Therefore, the following adaptation of customary procedures was undertaken.

### METHOD

One hundred grams of liver extract (Lilly, for oral use) was dissolved in 1 liter of water. Lead acetate in excess of that needed for complete precipitation was added, and the precipitate filtered off and discarded. The filtrate was treated with hydrogen sulfide to remove the excess of lead. The lead sulfide was washed once by shaking up with 1 liter of water and filtering. The combined filtrates were evaporated under reduced pressure to a volume of 1 liter. Then for each 100 c.c. of liquid, 17.5 c.c. of concentrated hydrochloric acid (sp. gr. 1.180) was added, and the mixture autoclaved for one to one and one-half hours at 60 pounds pressure. (This treatment destroys possible interfering substances.<sup>4, 5</sup>) After the liquid has cooled somewhat it was filtered and then cleared by boiling repeatedly with animal charcoal and filtering. (The animal charcoal is first thoroughly extracted with boiling water.) The clear, almost colorless liquid was evaporated to a thick syrup

\*From the Department of Medicine, University of Chicago.  
Received for publication, March 15, 1938.

under reduced pressure. The syrup was repeatedly extracted with hot 95 per cent alcohol, leaving a residue of inorganic matter. The hot alcoholic extracts were filtered through a hot water funnel and then treated with an excess of mercuric chloride in alcoholic solution. A voluminous white precipitate formed at once and was filtered off after standing twenty-four hours. It was washed with a little alcoholic bichloride solution, then suspended in 2 liters of hot water and treated with hydrogen sulfide to saturation. The mercuric sulfide was filtered off and washed with water. The combined filtrates were evaporated to a syrup under reduced pressure and again extracted with hot 95 per cent alcohol. The alcoholic solution was evaporated to a thick, light amber gum. The gum was taken up in a little water, in which it is very soluble, and treated with an equal volume of concentrated hydrochloric acid. This mixture was poured into a concentrated solution of gold chloride in 3 N hydrochloric acid. A voluminous bright yellow precipitate formed at once. It was recrystallized from its mother liquor by warming, then filtered off, washed with a little water, and dried in the oven at 105° C. After cooling in a desiccator, it weighed 2.4 gm. and melted to a red liquid with the evolution of gas at 253° C. with moderately rapid heating. This gold salt was recrystallized four times from dilute hydrochloric acid containing a little gold chloride. The melting point remained the same. This material was then analyzed for its elements. There were found: C 13.60 per cent, H 3.07 per cent, N 3.02 per cent, and Au 44.39 per cent. These values correspond well with the formula:  $C_5H_{14}NOCl \cdot AuCl_3$ , the chloroaurate of choline, for which is calculated: C 13.54 per cent, H 3.16 per cent, N 3.16 per cent, and Au 44.47 per cent. Further recrystallizations of the gold salt from water and from dilute hydrochloric acid did not change the melting point, if the same rate of heating was observed in all tests.

A sample of choline hydrochloride (Pfaustiehl) was converted into its chloroaurate, which was then recrystallized twice from dilute hydrochloric acid containing a little gold chloride. The manner of crystal formation and the crystals were like those in the experiments with the material from liver.

The mixed melting point determination was made as follows: A sample of the aurate from liver and a sample of the aurate from choline were dried in the oven and cooled in the desiccator at the same time. One capillary tube was used for each salt alone, and one for an intimate mixture of the two. All tubes were of the same size and thickness and filled to the same depth and degree of packing. All three were then attached to the same thermometer and heated in the same bath together. The behavior was identical for all three: with slow heating, softening and reddening began at 248° C., and melting was complete at 250° C. All formed red liquids with the evolution of gas.\*

#### COMMENT

Although the presence of choline in liver was demonstrated many years ago,<sup>6</sup> it was surprising to find it representing the nitrogenous bases in liver

\*These precautions are necessary because the melting point of choline chloroaurate has been given at anywhere from 238° to 256° C. It seems to vary with the mode of preparation of the salt, the thickness and packing in the tubes, and the rate of heating, etc.

extracts which are prepared by methods that should have separated it because of its solubility in both water and alcohol. The rather remote possibility of an effect on pernicious anemia is being investigated.

### SUMMARY

A study of liver extract for its content of nitrogenous bases showed the presence of at least 1 per cent of choline.

### REFERENCES

1. Jacobs, H. R.: On the Nature of the Antipernicious Anemia Principle III. The Response of a Case of Pernicious Anemia to the Oral Administration of a Tyrosinase-Tyrosine Mixture, *J. LAB. & CLIN. MED.* 22: 892, 1937.
2. Klein, G., Krisch, M., Pollauf, G., and Soos, G.: Zum mikrochemischen Nachweis der Betaine in der Pflanze. Glykokollbetain, Stachydrin und Trigonellin (gleichzeitig ein Beitrag zum Nachweis von Cholin und Nikotinsäure), *Oesterr. botan. Zeits.* 80: 273, 1931.
3. Cohn, E. J., Minot, G. R., Alles, G. A., and Salter, W. T.: The Nature of the Material in Liver Effective in Pernicious Anemia, *J. Biol. Chem.* 77: 325, 1928.
4. Nottbohm, F. E., and Mayer, F.: Ueber das Vorkommen und den Nachweis von Cholin im Kaffee, *Ztschr. f. Untersueh. d. Lebensmitt.* 63: 176, 1932.
5. Andrlík, K., Velich, A., and Stanek, V.: Das Betain in physiologischemischer Beziehung, *Zentralbl. f. Physiol.* 16: 452, 1902.
6. Smorodinzew, J.: Ueber die stickstoffhaltigen Extraktstoffe der Leber, *Zeits. f. physiol. Chem.* 80: 218, 1912.

## STUDIES ON THE MECHANISM OF LEUCOCYTOSIS

ANDERSON NETTLESHIP, NASHVILLE, TENN.\*

THESE experiments were designed to test two of the possible causes of leucocytosis: withdrawal of leucocytes from the blood stream or the presence of an irritant in the blood. The first of these was tested by the withdrawal of leucocytes by hemorrhage and by their withdrawal into an abscess area. The second idea was attacked through the use of substances which can be readily followed in the tissue where they were injected and in the blood.

### EXPERIMENTS

The usual blood techniques (lateral ear vein blood) were employed; cover slip smears were stained with Wright's stain. Chinchilla rabbits with a normal blood picture were used throughout. Total and differential counts were done for two days previous to as well as directly before any experimental procedure. Following the initiation of the experiment counts were done at two- to four-hour intervals for twenty-four hours, eight to twelve hours after.

*Withdrawal Experiments.*—1. Ten animals comprised this group. After establishing their blood cytologic picture as normal, quantities of blood ranging

\*Fellow in Medicine, the National Research Council.  
Received for publication, March 15, 1938.

from 4 to 14 cc were removed by venepuncture from the femoral vein, care being exercised not to traumatize tissues. No animal showed a leucocytosis, even though as many as 140 million leucocytes were removed with 14 cc of blood (the count being 10 thousand)

2 In inflammation leucocytes are withdrawn from the circulation into the inflammatory area. In six animals abscesses were produced by the intra cutaneous injection of streptococci. White counts were then made at the usual intervals. After each count, the injected area from one animal was removed for sectioning. Caution was used in having the abscess sectioned as nearly as possible through its exact center. The leucocytes in such a section were counted by starting at one edge of the abscess and progressing in parallel fields. The volume of the tissue in the section was then determined. Considering the abscess to be lenticular in shape which is usually the case, the total number of leucocytes in the whole abscess was fairly accurately determined. Using this method, the total number of leucocytes in the abscesses was found to be

	Time After Streptococcus Injection	
	2 Hours	24 Hours
Leucocytes in abscess	978,000	122 million
Leucocytosis increase per cent above normal	30	150

At twenty four hours the total circulating leucocytes [with a leucocytosis of 25 thousand (average for this time) and an animal weighing 2,000 gm] number some 5 billion. It is difficult to believe that 122 million leucocytes in an abscess could cause over 5 billion to appear in the circulation, so that withdrawal, either by venepuncture, which gives no leucocytosis, or into an abscess, cannot account for leucocytosis.

These experiments point to the presence of some substance in the blood which brings about leucocytosis.

*Experiments to Test for the Presence of the Injected Substance*—1 Silver nitrate. Fourteen animals were used. Four control animals were injected intra cutaneously with 25 cc of distilled water, they remained normal. The other animals were injected with 25 cc of a 10 per cent silver nitrate solution. Almost immediately edema and reddening appeared at the injection site. This reaction progressed during the first twenty four hours. By the seventy second hour necrosis appeared in the center. This spread and by the fourth day was as large as 2 by 2.5 cm. The blood picture of these animals changed rapidly. They showed a leucopenia within four to eight hours after injection. In one animal the drop was from its normal of 9,000 to 2,300. Following primary leucopenia, which lasted twenty four to forty eight hours, there was a marked leucocytosis. Counts as high as 19,000 were common. This lasted five to ten days. The leucopenia and leucocytosis were due to variations in polymorphonuclear leucocytes. Chart 1 shows a typical curve.

Silver determinations were done on the femoral vein blood, femoral bone marrow, sternal bone marrow, spleen, liver, lungs, and skin. The tissue or blood was obtained at two, eight, fourteen, and thirty-six hours, four, seven, and nine day intervals. The blood or organ is ashed and treated with hydro



chloric acid. After digestion with zinc and filtering, the residue is treated with nitric acid. The addition of sodium chloride solution gives a positive test of abundant white precipitate (silver chloride) provided silver is present. This method is sensitive to one part in 800,000 (Benedict<sup>1</sup>). No positive tests were obtained on either the blood or any internal organ. Skin sections from the injected areas invariably showed large quantities of silver.

2. Egg albumen. Twelve animals were used, three as controls which remained normal. Nine animals were injected with sterile crystalline egg albumen intracutaneously, with 4.0 c.c. doses, at four-day intervals, five injections being given to each animal. (The egg albumen was prepared freshly by the addition of ammonium sulfate and recrystallization. The salt was dialyzed off and the solution sterilized with a Berkefeld filter. Micro-Kjeldahl determinations for nitrogen placed the protein content at 4.5 per cent.) Following each of the sensitizing doses, a leucopenia set in within

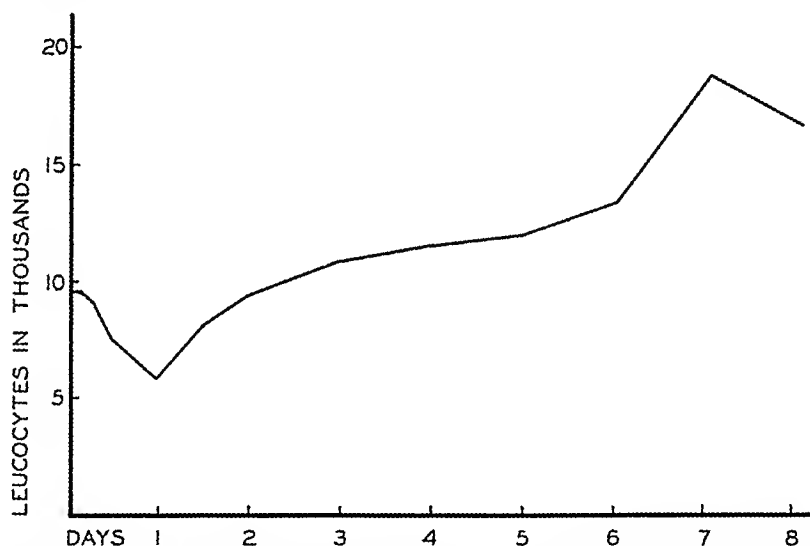


Chart 1.—This chart shows the leucocyte curve obtained after the intracutaneous injection of 2.5 c.c. of sterile silver nitrate. Note the primary leucopenia and the relatively late leucocytosis. This is a typical curve.

two hours and lasted twelve hours. The lowest count so obtained was 3,800; this animal's normal was 9,400 just prior to injection. The leucopenia was less with each successive dose. The urine was analyzed for albumin during these injections and was found to be present in abundance.

Following the last sensitizing dose, fourteen days were allowed to elapse. Into a new skin area, 2 c.c. of albumen were then injected. This injection caused a typical Arthus reaction. Reddening and edema set in early, and necrosis appeared about the fourteenth hour. The necrosis spread up to the seventy-second hour. The leucocyte study during the production of the lesion is shown in Chart 2. A severe primary leucopenia (polymorphonuclear cell loss) set in within two hours, reaching its lowest by the sixth hour. In between twelve and twenty-four hours the cell count returned to normal, to increase to 200 per cent or more above normal by the seventy-second hour.

This leucocytosis lasted three to five days. The highest count obtained was 47,000. By the third day there was bone marrow hyperplasia. The leucocytosis was caused by an increase in polymorphonuclear cells. The data below show the averages, in thousands, for the animals.

Directly Before Injection		Injection		Following Injection			
10 hr	10 hr	24 hr	48 hr	2 hr	72 hr	96 hr	120 hr
4.5	4.7	10.5	21.1	5.6	21.0	17.6	15.1

After the necrosing injection, the urine of these animals was tested for albumin, none could be found in the highly sensitized animals' urine.

In summary, no silver nitrate or egg albumen could be detected in the blood during leucocytosis. With these two substances peripheral necrosis is apparently necessary for the production of leucocytosis.

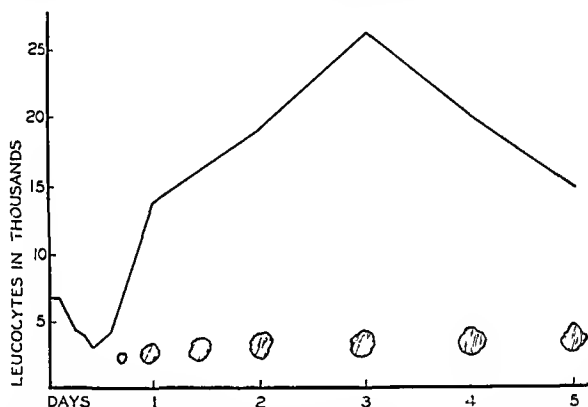


Chart 2—This chart shows a typical leucocyte curve obtained after injection of the necrotizing dose of egg albumen. The stippled areas at the bottom show the gross necrosis. Notice that the first appearance of this is just at the time the leucocytosis sets in.

#### DISCUSSION

It is a common clinical observation that postoperatively or following massive hemorrhage, a leucocytosis occurs. However, in such observations there are always factors other than the hemorrhage itself to be considered, such as anesthesia, infection, trauma. Experimentally, Dawson<sup>2</sup> produced leucocytosis by massive hemorrhage, however, he introduced other factors. With caution 14 cc of blood were removed from a rabbit without producing a leucocytosis, and recently as much as 30 to 40 cc of blood has been taken from a single animal without causing a leucocytosis (Boyd et al<sup>3</sup>). This is comparable to a massive hemorrhage in man.

As we have seen, even though 122 million leucocytes go from the blood stream into an abscess in twenty four hours, the circulating leucocytes are 5 billion. This enormous increase is all out of proportion to what would be

expected if leucocytosis were produced by simple withdrawal. Even though the method is an approximation, the disproportion is still striking. In human beings, too, one is struck by the relatively small number of leucocytes, say in a furuncle, compared to the numbers such a lesion will call forth into the blood stream.

These observations suggest that there is some substance in the blood stream which brings about leucocytosis. The present experiments tested for the necrotizing substance itself (silver nitrate and egg albumen). None of either substance could be detected during leucocytosis. The criticism may be offered that small quantities of albumin may escape from the site of injection. In this regard Opie<sup>4</sup> remarks, speaking of antigen (egg albumen), "The same substances introduced in the immune animal are fixed at the site of entry and are not found in the blood." Even more against the idea that it is the albumin as such acting on the bone marrow to produce leucocytosis, is the fact that the leucocyte increase occurs parallel in time to the necrosis of peripheral tissue, and is in proportion to that necrosis. It may be that the inciting agents themselves do diffuse from their site of entry to cause leucocytosis. However, this could not be demonstrated with the agents used. The rapidity with which leucocytosis set in is rather against the idea. The present observations point to a correlation between the advance of necrosis and leucocytosis.

#### CONCLUSIONS

1. The withdrawal of leucocytes, either by venepuncture or into an abscess area, cannot account for leucocytosis.

2. In acute experiments silver nitrate injected intracutaneously cannot be found in the blood or internal organs. It is recoverable in large amounts from the area of injection.

3. Silver nitrate injected intracutaneously causes necrosis accompanied by a primary leucopenia followed by marked polymorphonuclear leucocytosis.

4. Egg albumen injected into non-sensitized animals diffuses from the injection site and causes a leucopenia.

5. Injected into sensitized animals, egg albumen does not diffuse into the blood stream. Peripheral necrosis occurs with a concurrent well-defined leucocytosis.

#### REFERENCES

1. Benedict, S. R.: Personal communication.
2. Dawson, P. M.: Effects of Venous Hemorrhage and Intravenous Infusion in Dogs, *Am. J. Physiol.* 4: 1, 1900.
3. Boyd, E. M., Stevenson, J. W., and Watkins, E. A.: Lipid Content of Blood Leucocytes Following Hemorrhage in Rabbits, *Proc. Soc. Exper. Biol. & Med.* 37: 653, 1938.
4. Opie, E. L.: The Fate of Antigen (Protein) in an Animal Immunized Against It, *J. Exper. Med.* 39: 659, 1924.

## ACHLORHYDRIA IN LEUCEMIA\*

OSCAR O. MEYER, M.D., MADISON, WIS

IN 1932 Strandell and Toigerstrand<sup>1</sup> reported a series of 22 cases of leucemia in which they found gastric achlorhydria to be of frequent occurrence. Particularly was this true in lymphocytic leucemia. These authors reported finding achlorhydria in 7 of 14 cases of myeloid leucemia and in 6 of 8 cases of lymphoid leucemia, and they wondered whether or not achylia was not responsible for gastric complaints which are so common in leucemia. The average age for the group with myeloid leucemia and achlorhydria was slightly younger, but the patients with lymphoid leucemia and achlorhydria averaged more than 10 years older than those with free acid.

All of the gastric analyses in their series were done after administration of an Ewald meal. The stimulus to secretion afforded by the administration of histamine was not utilized. Bloomfield and Pollard<sup>2</sup> emphasize that achlorhydria cannot be accepted as definitely existing unless repeated samplings after histamine administration prove negative for free hydrochloric acid.

Since about the time the report of Strandell and Toigerstrand<sup>1</sup> appeared, gastric analyses have been made on nearly all of the cases of chronic leucemia admitted to the medical service of the State of Wisconsin General Hospital. These cases, together with the few in which gastric analyses had incidentally been made previous to 1933, are included. If free acid was not demonstrated by a single aspiration after an Ewald meal, 1 mg. of histamine was administered subcutaneously to all cases.

### RESULTS

*Chronic Myeloid Leucemia*—Gastric analysis was done in 21 cases. Of these only 3, or 13 per cent, of the cases that received histamine, showed absolute achlorhydria. Seven other cases showed an absence of free acid after Ewald meal, but in both of the 2 cases of this group that later received histamine, free acid was found. It is interesting that of the 3 cases with histamine achlorhydria, 1 was a youth of 16. The ages of the other 2 patients were 43 and 56. The average age of the entire group of 21 patients was 44 years.

*Chronic Lymphoid Leucemia*—Gastric analysis was done in 19 cases. Of these 10, or 53 per cent, had achlorhydria after histamine administration. Four others had achlorhydria after Ewald meal alone, but when histamine was administered, 3 of these 4 demonstrated the ability to secrete hydrochloric acid. Of the 10 truly achlorhydric subjects, 1 was 38 years old, but the remainder ranged in age from 55 to 75 years. The average age of the group of 19 patients was just under 60 years.

\*From the Department of Medicine, University of Wisconsin Medical School.  
Received for publication March 16, 1938.

*Aleuemic Leucemia.*—The diagnosis in this group was established in most instances by lymph node or bone marrow biopsy. There were 10 cases. Only 3 patients had achlorhydria, and each of these had received histamine. The ages were 53, 55, and 62 years, respectively. The average age of the group of 10 patients was 51.5 years.

*Acute Leucemia.*—Only 5 cases were available in this group, 4 of lymphoblastic and 1 of myeloblastic leucemia, and consequently the figures can mean little. However, 2 of the cases, both lymphoblastic, showed achlorhydria after histamine. These patients were 49 and 64 years of age.

#### COMMENTS

Each of these series of cases is small, but they show, in some degree, the trend. Cognizance of the age periods for each group must be taken for as Bloomfield and Polland<sup>2</sup> and Vanzant and others<sup>3</sup> have shown, achlorhydria is increasingly frequent with greater ages. This fact must be recognized, particularly in the group with chronic lymphoid leucemia, where the average age is 60 years. In this group there is a high incidence of achlorhydria, 53 per cent. At this age period one would expect, in healthy individuals, an incidence of achlorhydria of approximately 25 per cent. Consequently, in these cases of chronic lymphoid leucemia, the frequency of achlorhydria appears to be significant.

In the patients with myeloid leucemia, a younger group, an incidence of achlorhydria of 13 per cent is not significant for at the average age of 44 years, this or a greater frequency might be anticipated in any group of individuals. In one case, a youth of 16, persistent achlorhydria was found. The significance of this, however, was not demonstrated as post-mortem examination was not permitted and, therefore, gastric disease was not excluded. The frequency of achlorhydria in the aleuemic leucemia group was 33 per cent. If the series comprised more than 10 cases, the results might be significant, but under existing circumstances, with an average age of 51.5 years, it cannot be so considered, and the suggestion is gained that the incidence of achlorhydria is not unusual. The smallness of the group with acute leucemia likewise permits no significant deduction relative to the incidence of achlorhydria.

It is noted that the incidence of achlorhydria in both myeloid and lymphoid leucemia is distinctly less than reported by Strandell and Torgersrud.<sup>1</sup> This can be explained by their failure to use histamine. Confirmation for this stand is offered by the several observations reported here where free acid appeared only after histamine administration.

In several cases of all types repeated study of the gastric contents, months apart, was done, but in all instances the results were consistently positive or negative for the respective cases.

It was not possible to demonstrate any relationship between the level of the leucocytes or erythrocytes and achlorhydria.

#### SUMMARY AND CONCLUSIONS

Gastric analysis after histamine administration was done in patients with leucemia. The incidence of achlorhydria in the various types of leucemia was as follows: Chronic myeloid leucemia 13 per cent, chronic lymphoid leucemia

53 per cent, aleucemic leucemia 33 per cent. The average ages of the groups was 44, 60, and 51.5 years in the order named. Two of 5 patients with acute leucemia had achlorhydria.

The incidence of achlorhydria is not significant in chronic myeloid leucemia nor, probably, in aleucemic leucemia. The frequency of achlorhydria in chronic lymphoid leucemia apparently is significant even though this disease occurs in an older age group. The explanation for this finding in chronic lymphoid leucemia is not as yet available.

#### REFERENCES

- 1 Strandell, B., and Torgersrud, T. Die Magensekretion bei Leukämie, *Acta med Scandinav Supp* 50, 344, 1932.
- 2 Bloomfield, A. L., and Pollard, W. S. *Gastric Acidity*, New York, 1933, Macmillan Co.
- 3 Vanzant, F. R., Alvarez, W. C., Eusterman, G. B., Dunn, H. L., and Berkson, J. The Normal Range of Gastric Acidity From Youth to Old Age. An Analysis of 3,746 Records, *Arch Int Med* 49: 315, 1932.
- Vanzant, F. R., Alvarez, W. C., Berkson, J., and Eusterman, G. B. Changes in Gastric Acidity in Peptic Ulcer, Cholecystitis, and Other Diseases Analyzed With the Help of New and Accurate Technique, *Arch Int Med* 52: 616, 1933.

## THE ADSORPTION OF PHENOL BY PROTEIN IN PREPARATION OF CERTAIN BIOLOGIC PRODUCTS\*

EUGENE CARDONE AND ROBERT MAZZARELLA, NEW YORK, N. Y.

#### INTRODUCTION

THE refining and concentration of antisera and antiplasmas involve in general terms the precipitation of different protein fractions by the addition of salts, by suitable dilution with water at definite hydrogen ion concentrations, and by other methods. In the processing of these materials in order, if possible, to maintain sterility, or at least to reduce bacterial growth to a minimum, preservatives are generally added. Results will be presented which show that when phenol is used for this purpose, the protein precipitates may carry down or adsorb considerable amounts of phenol. This factor must be considered before the addition of phenol to the final product in order to avoid excessive amounts of this preservative.

#### EXPERIMENTAL METHODS

*Phenol Estimations*—“The method of analysis consisted in the quantitative formation of tribromophenol by the addition of a bromide bromate solution and titration of the excess bromine with thiosulphate after the addition of potassium iodide, hydrochloric acid, and starch. The method was used first by Koppeschaal,<sup>2</sup> studied further by a number of workers, and a careful reinvestigation was given in detail by Scott<sup>3</sup> several years ago.

\*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.

Received for publication March 21, 1938.

†The following description is taken from the paper by McGuire and Falk.<sup>1</sup>

"In view of Scott's extended study and detailed presentation of the method, it will not be repeated here. For the removal of protein, the well-known Folin-Wu precipitation was used. To 5 c.c. of the material, 35 c.c. of water (or saline) were added, then 5 c.c. of 10 per cent sodium tungstate solution and 5 c.c. of 0.67 N sulphuric acid. The mixture was shaken, allowed to stand at room temperature for thirty minutes, and filtered through paper. To 40 c.c. of the filtrate, 160 c.c. water were added, and the phenol estimation carried out.

"Duplicate determinations as a rule agreed within 0.5 per cent of the amount of phenol found. Using the purest phenol obtainable, satisfactory recoveries, within 2 per cent, of the amount added, were obtained."

*Refining and Concentrating Antiserums.*—The precipitation of proteins in the refining of the antiserums as carried out in this Laboratory follows essentially the procedure described by Klein, Greenwald, and Falk,<sup>4</sup> with certain modifications to be described in detail in another connection. The following main points are to be considered in their bearing on the present problem. The designation of the successive steps follows the description given in the former paper.

b. *Dialysis of Serum.*—A definite concentration of phenol was added to the original serum. A considerable part of this phenol content was removed from the serum as a result of dialysis. At the conclusion of the dialysis, a certain amount of precipitate was present.

c. "*Acid Protein*" *Precipitation.*—The dialysate, after the addition of sodium chloride to N/20 and 0.3 per cent phenol, was brought to pH 5.1. The precipitate was removed by centrifuging.

e. *Precipitation of Antibacterial Substance.*—The liquid was brought to the desired pH (as determined in preliminary tests; either 5.9, 6.1, or 6.8), four volumes of distilled water containing 0.2 per cent phenol added. After standing overnight in the cold, the precipitate was removed by centrifuging.

f. *Solution of Antibacterial Substance.*—The precipitate was dissolved in the requisite amount of solvent containing sodium chloride and phenol. Phenol estimations were carried out at the following stages:

b. Original serum; serum after dialysis; centrifuged precipitate; supernatant.

c. Centrifuged acid precipitate; supernatant.

e. Centrifuged antibacterial precipitate; supernatant.

f. Final product.

The solid contents, dried at 110° C., of the precipitates in b, c, and e, were determined, and the amount of phenol adsorbed by the protein calculated. This calculation includes correcting for the supernatant liquid and its phenol, protein, and sodium chloride contents present in the moist centrifuged precipitates.

When phenol was to be added directly to antibacterial preparations, it was mixed with ether in the proportion of three parts of the former to four parts of the latter.

*Experimental Results.*—The phenol contents are given as weights per cent per 100 ml. of liquid or precipitate. For example, 0.3 per cent phenol signifies

0.3 gm phenol per 100 ml of liquid or precipitate (volume of precipitate as measured in calibrated centrifuge tubes)

The following typical experiments are presented

#### Experiment 1 Antipneumococcus (Type II) Preparation

	Per cent Phenol
Original serum (1040 ml)	0.262
Mixture after dialysis (1140 ml), after sampling (1020 ml) centrifuged	0.094
Supernatant (938 ml)	0.092
Precipitate moist (82 ml)	0.120
Precipitate dried (14 gm)	0.236
Sodium chloride and phenol (approx 0.3 per cent) added to mixture	
Mixture before acid protein precipitation (1010 ml)	0.380
Acid protein precipitation—pH 5.1 (volume loss due to manipulations)	
Supernatant (900 ml)	0.378
Precipitate acid protein moist (20 ml)	0.488
Precipitate acid protein dried (1.6 gm)	1.750
Supernatant—pH 5.9—4 volumes distilled water containing 0.2 per cent phenol	
Supernatant (4100 ml)	0.240
Precipitate—antibacterial protein moist (68 ml)	0.344
Precipitate—antibacterial protein dried (15.4 gm)	0.697

The antibacterial moist precipitate after centrifuging was 68 ml in volume and 0.344 per cent in phenol content, containing, therefore, 0.234 gm of phenol. The volume of the original serum was 1,040 ml. If the final volume of the preparation were to be one sixth of the initial volume, or 173 ml, the moist precipitate would be diluted to that volume with salt solution of suitable concentration. In order to obtain the proper phenol concentration, a calculated amount would be added. If the final volume were to be 173 ml as just indicated, and if 0.5 per cent phenol concentration were desired, 0.865 gm would be added. Actually then, because of the phenol present in the moist precipitate, the phenol content would be 0.635 per cent (instead of the desired 0.5 per cent). If 0.35 per cent phenol concentration were desired in the final product, 0.606 gm would be added, but this would give a concentration of 0.486 per cent. If the final volume of the preparation were to be one twelfth of the initial volume, or 87 ml, the amount of phenol calculated to give 0.5 per cent would give an actual concentration of 0.769 per cent, and the amount calculated for 0.35 per cent an actual concentration of 0.620 per cent.

#### Experiment 2 Antipneumococcus (Type II) Preparation

	Per cent Phenol
Dialyzed serum after addition of 0.3 per cent phenol	0.346
Supernatant of acid protein precipitate	0.320
Acid protein precipitate moist	0.432

#### Experiment 3 Antipneumococcus (Type VIII) Preparation

	Per cent Phenol
Original serum	0.298
Serum after dialysis—containing precipitate	0.098
Supernatant of dialyzed serum	0.088
Moist precipitate of dialyzed serum	0.222
Mixture containing antibacterial precipitate pH 5.9	0.233
Moist antibacterial precipitate	0.234



## Experiment 4. Antipneumococcus (Type V) Preparation

Serum after dialysis and acid protein precipitation	Per cent Phenol 0.368
Antibacterial substance precipitated at pH 6.1 by addition of 4 volumes of distilled water containing 0.2 per cent phenol	
Supernatant of antibacterial precipitate	0.232
Moist antibacterial precipitate	0.342

Some results on the phenol contents of preparations before and after dialysis (in cellophane bags against running water for six days) are presented in Table I. In every case phenol was present after dialysis in concentrations ranging from 0.032 to 0.118 per cent.

TABLE I

EXPERIMENT NO.	MATERIAL	VOLUME ML.		PER CENT PHENOL	
		INITIAL	FINAL	INITIAL	FINAL
1	Antipneumococcus serum	1,040	1,140	0.262	0.094
2	Antipneumococcus serum	18,600	22,000	0.246	0.032
3	Antipneumococcus serum	19,500	23,000	0.298	0.098
4	Antipneumococcus serum	20,000	23,000	0.296	0.094
5	Antistreptococcus serum	720	770	0.482	0.118
6	Refined antipneumococcus preparation			0.504	0.070
7	Pneumococcus antibacterial substance containing 0.5 per cent phenol, precipitated by 50 per cent saturation with ammonium sulfate solution		1,250		0.048

## DISCUSSION

It may be stated that the estimation of the phenol in the mixtures is accurate to 2 per cent of the amount present or better. The calculation of the concentrations, however, should be defined more clearly than is the case generally. In the analytical determinations presented here, per cent phenol was given as grams per 100 c.c. of solution, mixture, or precipitate. On the other hand, when added to serum in the routine procedures, the addition of phenol is calculated as per cent by volume; that is, nine parts of melted phenol are diluted with one part of water, and this solution is considered 90 per cent phenol by volume. If it is desired, for example, to have 1,000 c.c. of serum containing 0.2 per cent phenol by volume, it would be necessary to add 2.22 ml. of the 90 per cent phenol solution, which would correspond to 2.0 ml. phenol or 2.13 gm.

The results show clearly that phenol is carried down, or adsorbed, by precipitating protein to a concentration at least equal to, and generally considerably greater than, that present in the solution.

It will hardly be necessary to discuss the experimental findings in detail. Antipneumococcus preparations were mainly used in this study because they were available in considerable numbers at the time. There is every reason to believe that other antibody preparations, protein materials of different kinds, and even nonprotein material, such as aluminum hydroxide, when precipitated from solutions containing phenol, would carry down appreciable quantities of phenol which must be reckoned with in the later manipulations.

The data for Experiment 1 are given in fairly complete form. It is evident that the precipitate from dialysis, the acid protein precipitate, and the antibacterial protein precipitate, carry down phenol in concentrations greater than would be expected from its concentration in the solution. Calculations are given to show the amounts of phenol present in the final preparations if the usual method, as indicated earlier in this paper, of adding additional phenol is followed. Experiments 2, 3, and 4 show similar results in more condensed form.

In the dialysis of the various protein preparations, it is generally assumed that phenol is removed in the course of the dialysis. The data from Experiments 1 to 7 show that this is not entirely correct. Even after six days of dialysis, considerable concentrations of phenol may be retained, between 0.032 and 0.118 per cent in the results given. This is of importance in connection with the precipitation of antitoxin and antibacterial proteins by ammonium sulfate (or other precipitant) where, if phenol is present, it would in all probability be adsorbed by the precipitating protein. The phenol would not be removed completely in the subsequent dialysis, and should be considered when adding preservative to the desired concentration.

In view of the experiments described in this paper it may be stated that the phenol content of protein (and other) materials containing phenol as preservative should be controlled during their refining and concentration. This is especially important for the antitoxin and antibacterial preparations intended for therapeutic use.

#### SUMMARY

The phenol contents of a number of antibacterial preparations at various stages of refining and concentration were determined. It was found that considerable concentrations of phenol were carried down, or adsorbed, by the separated proteins. Special care must, therefore, be exercised in the addition of phenol to the final therapeutic products.

#### REFERENCES

- 1 McGuire, G., and Falk, "Biological Products of Phenols and Cresols Added to", *CLIN. MED.* 22, 641, 1937.
- 2 Koppeschaar, W. F., "des Phenols, *Ztschr. f. anal. Chem.*" 15, 233, 1876.
- 3 Scott, R. D., "Application of a Bromine Method in Determination of Phenol and Cresols, *Indust. & Engin. Chem., Analyt. Ed.* 3, 67, 1931.
- 4 Klein, A. J., Greenwald, C. K., and Falk, K. G., "The Refining of Antiserums, *J. LAB. & CLIN. MED.* 19, 1324, 1934.

# EFFECT OF HEAT ON THE HEMOLYTIC AND SKIN-NECROTIZING FACTORS IN STAPHYLOCOCCUS TOXIN<sup>a</sup>

R. H. RIGDON, M.D., NASHVILLE, TENN.

MANY observations have been made on the effect of heat on staphylococcus toxin; however, these usually have been limited to determining whether or not a fraction of the toxin has been destroyed at a specific temperature after a definite interval. Systematic studies on the effect of heat on many of the hemolysins have been made by Madsen and his co-workers. Included in their group are vibrolysin, tetanolysin, streptolysin, and epeiralyisin.<sup>1</sup>

The present investigation was undertaken following the suggestion of Dr. Thorvald Madsen. The methods used are essentially the same as those used by him in observing the effect of heat on hemolysins.<sup>2</sup> This study shows the effect of heat on the hemolytic and skin-necrotizing factors in staphylococcus toxin.

## METHODS AND MATERIAL

The staphylococcus toxin was supplied through the courtesy of Lederle Laboratories and was prepared as follows: Cultures of *Staphylococcus aureus* were inoculated on a semisolid agar under a mixture of 20 per cent carbon dioxide in oxygen for forty-eight hours at 37.5° C. The medium contained 1000 c.c. veal infusion, 2.0 per cent Difco proteose peptone, 0.5 per cent sodium chloride, 0.7 per cent sodium acetate, and 0.2 per cent agar. The pH was adjusted to 7.4.

After incubation, the agar was separated from the liquid by filtration in the cold, and the filtrate was passed through a Mandler filter to render it bacteria free.

The toxin was preserved with merthiolate in a final dilution of 1:10,000, and at the time of preparation had the following potency: hemolytic titer 1:900, dermonecrotic titer 1:800, and acute killing dose (intravenous) 0.05 c.c.

The water bath was thermoelectrically controlled. The temperature never varied more than 0.02 to 0.08° C. during any experiment. The temperature at which the toxin was heated is given in the experiment.

Approximately 40 c.c. of the staphylococcus toxin was put into a pyrex glass cylinder. It was then placed in the water bath. A Beckmann thermometer and a pyrex glass stirring rod were placed in the toxin through two holes in the stopper. An electric motor was used to stir the toxin at a constant speed. When the temperature of the toxin in the cylinder reached the temperature of the water bath, 2 c.c. were removed. At definite intervals, usually ten minutes, subsequent samples of toxin were taken. Some of the experiments ran as long as one hundred and fifty minutes. The samples of toxin were placed immediately in an ice bath (-0° C.). The toxin froze after a short time. The tubes

<sup>a</sup>From the Department of Pathology, Vanderbilt University Medical School, Nashville. Received for publication, March 24, 1938.

were then placed in the icebox (5° C), and usually kept for twenty four to seventy two hours before the hemotoxin titrations were made

Rabbit's red cells were used for the indicator in the hemolytic test. The cells were washed three times in physiologic saline and suspended in a similar salt solution. One cubic centimeter of a 2 per cent suspension of these cells was added to each tube.

TABLE I

AMOUNT OF STAPHYLOCOCCUS TOXIN NECESSARY TO PRODUCE THE SAME DEGREE OF LYSIS AT ANY OF THE FOLLOWING TEMPERATURES AND THE RESIDUAL HEMOTOXIN EXPRESSED IN PER CENT

TIME MINUTES	45° C		47.4° C		52° C		55° C		54.03° C	
	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY
0	0.000126	100.0	0.000126	100.0	0.000126	100.0	0.000126	100.0	0.000267	47.3
10	0.000128	95.4	0.000127	100.0	0.000143	88.1	0.000326	38.6	0.000623	20.2
20	0.000138	91.3			0.000164	76.8	0.00044	28.7	0.001216	10.3
30	0.000128	95.4	0.000110	78.7	0.000234	57.8	0.00074	16.9	0.002044	5.7
40	0.000128	95.4			0.000244	51.6	0.000883	14.2	0.003833	3.2
50	0.000136	92.6	0.000153	82.3			0.001193	10.5	0.006266	2.0
60	0.000132	95.4			0.000365	34.5	0.001700	7.4	0.008233	1.5
70	0.000153	82.3	0.000210	60.0	0.000568	22.0	0.002466	5.1	0.009330	1.3
80	0.000155	81.2			0.000577	21.8	0.002866	4.3	0.013330	0.94
90	0.000138	91.3	0.000206	61.6	0.000676	18.6	0.003600	3.5	0.015660	0.80
100	0.000145	86.9			0.000825	15.3	0.004100	3.07	0.019660	0.64
110	0.000136	92.6	0.000226	54.8	0.000833	13.8	0.005100	2.4	0.024000	0.52
120	0.000138	91.3			0.000900	14.0	0.008660	1.4	0.025600	0.41
130			0.000195	64.6						
140										
150	0.000135	93.3	0.000230	54.7						
	54.05° C		55° C		55.65° C		56.35° C		57° C	
	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY	TOXIN IN CC	% RESIDUAL TOXICITY
0	0.000300	42.0	0.000633	19.8	0.001500	8.4			0.001206	9.9
10	0.000766	16.4	0.001600	7.8	0.008930	3.2	0.004500	2.8	0.006160	2.0
20	0.001580	7.9	0.004110	3.06	0.007560	1.6	0.009000	1.4	0.014500	0.87
30	0.002433	5.1	0.008260	1.5	0.011600	1.0	0.012000	1.0	0.027000	0.46
40			0.011430	1.1	0.013300	0.94	0.017500	0.77	0.034330	0.36
50			0.015000	0.84	0.017300	0.78	0.024000	0.52	0.049000	0.25
60			0.017160	0.73	0.019839	0.68	0.027000	0.46	0.060000	0.21
70	0.008060	1.5	0.026580	0.47	0.022160	0.56	0.031000	0.40	0.060000	0.21
80	0.013000	0.97	0.027160	0.46	0.028660	0.44	0.040000	0.31	0.070000	0.18
90	0.018580	0.67	0.035800	0.35	0.026160	0.48	0.042500	0.29	0.067500	0.17
100	0.021260	0.58	0.046600	0.27	0.033660	0.37	0.046500	0.27	0.090660	0.13
110	0.023160	0.54	0.044000	0.28	0.032330	0.38	0.056500	0.22	0.103300	0.11
120	0.026000	0.48	0.046330	0.27	0.042200	0.29	0.063000	0.2	0.105000	0.11
130							0.075000	0.168		
140							0.080000	0.157		
150										

The toxin was titrated over a range varying from 0.8 cc to 0.0001 cc. It was diluted with physiologic saline. The volume in each tube was kept constant by the addition of a 0.85 per cent solution of sodium chloride. The test tubes were uniform in size and measured 1 by 10 cm. The red cells were added to the dilution of toxin, and the tubes were kept at room temperature (27° to 32° C) overnight before the degree of lysis was recorded.

The amount of lysis which occurred in this period was determined by quantitative methods. Usually three tubes which showed only a small amount of

hemoglobin were selected for the standards from the series heated for the shortest period at each of the different temperatures. The amount of hemoglobin in each tube in all the series of titrations heated at the same temperature was compared with these standards. The average of the amount of staphylococcus

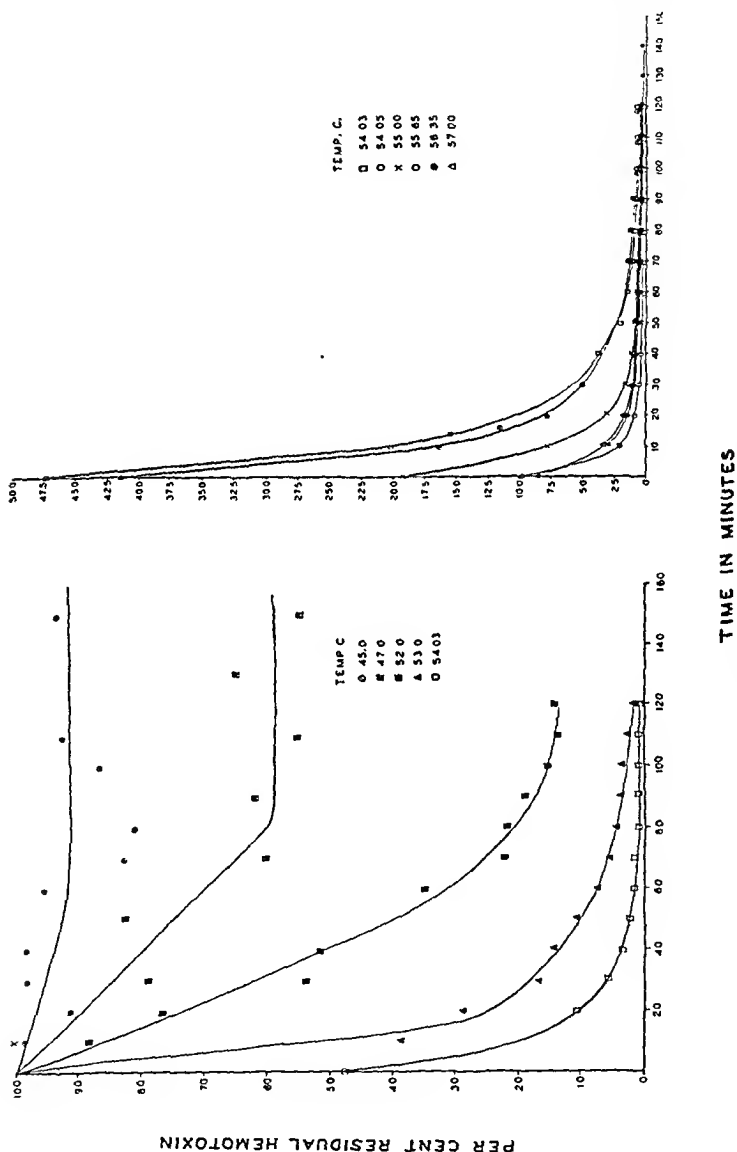


Chart 1.—The curves represent the per cent of residual hemotoxin plotted against time for the different temperatures. The two graphs are proportional. The data are taken from Table I.

toxin in the three tubes was taken in each series which matched the standards. This quantity of toxin was considered to be necessary to produce the same degree of lysis as that in the standard tubes for that particular temperature.

The percentage of residual hemotoxin in any sample was determined by dividing the volume of toxin in the specific tube into the amount of toxin

necessary to produce the degree of lysis which was considered to represent 100 per cent toxicity of the unheated toxin.

The amount of hemoglobin was determined by comparing directly the color in the different tubes.

The effect of heat on the skin-necrotizing factor in staphylococcus toxin was determined by injecting intradermally into two rabbits 0.1 c.c. of the different samples of the toxin in which the amount of residual hemotoxin was known.

TEMP.	TIME IN MINUTES	0	10	20	30	40	50	60	70	80	90	100	110	120
53°	% RESIDUAL HEMOTOXIN						10.5	7.4	5.1	4.3	3.5	3.07	2.4	1.4
	SKIN NECROSIS						•	•	•	•	•	•	•	X
54.03°	% RESIDUAL HEMOTOXIN			10.3	5.6	3.2	2.0	1.5	1.3	0.94	0.80			
	SKIN NECROSIS			•	•	•	•	X	X	X	X			
55°	% RESIDUAL HEMOTOXIN	19.8	7.8	3.06	1.5	1.1	0.84	0.73	0.47					
	SKIN NECROSIS	•	•	•	•	•	X	X	X					
57°	% RESIDUAL HEMOTOXIN	9.9	2.0	0.67	0.48	0.36	0.25	0.21	0.21					
	SKIN NECROSIS	•	•	X	X	X	X	X	X					

Chart 2.—Skin necrosis in the rabbit following the intradermal injection of staphylococcus toxin heated at the different temperatures for varying intervals of time compared with the residual hemotoxin. No necrosis occurred in the areas indicated by X.

#### EXPERIMENTAL

Table I shows the amount of staphylococcus toxin heated at temperatures between 45° and 57° C. that is necessary to produce the same degree of lysis for any specific temperature. It also gives the amount of residual hemotoxin expressed in per cent.

Chart 1 shows the results obtained when the residual hemotoxin is plotted against time in minutes.

The areas of skin necrosis produced by the different samples of toxin are shown in Chart 2. It can be seen from these data that the size of the necrotic areas are proportional to the amount of the residual hemotoxin. Only samples of toxin heated for different intervals at 53°, 54.03°, 55°, and 57° were used since only a limited number could be injected intradermally into a single animal.

#### DISCUSSION

The physicochemical views with regard to toxin and antitoxin advanced by Ehrlich,<sup>3</sup> Arrhenius and Madsen,<sup>4</sup> and Bordet,<sup>5</sup> about the beginning of the twentieth century, have brought forth considerable discussion. Our knowl-

edge of such processes even at present is very limited. Little has been contributed since 1900 as to the composition of toxins. Until more information is obtained with regard to these substances called toxins, it appears of little value to attempt to determine the type of reaction obtained when staphylococcus toxin is heated.

The data given in Table I show that staphylococcus toxin is thermolabile. Chart 1 shows that the rate of detoxification is influenced by the temperature and the length of time it is heated. The hemolysin is destroyed very rapidly during the first thirty minutes when the temperature is 53° C. or higher. The residual hemolysin at this time apparently is more resistant to heat. Only a slight decrease occurs in the amount of hemotoxin when this same toxin is heated at 45° for one hundred and fifty minutes.

The rate of detoxification of staphylococcus toxin by heat suggests that there may be two hemolytic agents present which vary in their thermolability.

Schrek,<sup>6</sup> in studying heat inactivation of tetanolysin, found that the first 99 per cent was inactivated at a constant rate and the last 1 per cent was inactivated slower. He suggested that the accumulation of the products of heat inactivation decreased the rate of the reaction.

It is of interest to note that the skin-necrotizing factor in staphylococcus toxin is destroyed by heat. The rate at which it is detoxified runs essentially parallel with the rate at which the hemolysin is destroyed. The results of the effect of heat on the hemolysin and skin-necrotizing factors in staphylococcus toxin suggest that they may be the same substance. Apparently no one has been able to determine conclusively that they are the same or different substances.

#### SUMMARY

The hemolysin and the skin-necrotizing factor in staphylococcus toxin are both detoxified by heat. The rate at which these fractions are destroyed is essentially parallel. It is suggested that they may be the same substance.

The rate at which the hemotoxin is destroyed is influenced by the temperature and the length of time the toxin is heated.

The rate of detoxification of staphylococcus toxin by heat suggests that there may be two hemolytic substances which vary in their thermolability.

I wish to express my appreciation to Dr. Arthur C. Omberg, Physicist in the Roentgenological Department of Vanderbilt University, for his assistance in this study, and to the Department of Physiology and Pharmacology which supplied the equipment for this experiment.

#### REFERENCES

1. Madsen, Thorvald: Influence of Temperature on Antigens and Antibodies, *J. State Med.* 31: 151, 1923.
2. Famulener, L. W., and Madsen, T.: Die Obschwachung der antigene durch Erwärmung, *Biochem. Ztschr.* 11: 186, 1908.
3. Ehrlich, P.: Ueber Toxine und Antitoxine, *Therap. d. Gegenw.*, 193, 1901.
4. Arrhenius, S. V., and Madsen, T.: Anwendunz der physikalischen chemie auf das Studium der Toxine und Antitoxine, *Ztschr. f. physiol. Chem.* 44: 7, 1903.
5. Bordet, J.: Les Serums Hemolytiques, Leurs Antitoxines, *Ann. Inst. Pasteur* 14: 257, 1900.
6. Schrek, Robert: Effect of pH on Heat Inactivation of Tetanolysin, *J. Immunol.* 25: 183, 1933.

# SIMULTANEOUS BILATERAL SPONTANEOUS PNEUMOTHORAX

## REPORT OF A RECURRENT CASE DUE TO CONGENITAL CYSTIC LUNG DISEASE

DLAN B. COLE, M.D., AND WALTER L. NALLS, M.D.  
RICHMOND, VA

IN 1934 Oechsli and Miles reported a case of recurrent simultaneous bilateral spontaneous pneumothorax due to congenital cysts of the lung. At the time of this report they found reported in the literature a total of 77 cases of bilateral spontaneous pneumothorax, two of which were due to cystic lung disease. Ghekman and Sehlomovitz, in 1936 also reviewed the literature on simultaneous bilateral pneumothorax and reported a total of 82 cases, three of which were due to congenital cystic disease of the lungs. We wish to add another case of this rare entity, the diagnosis of which was made before death and confirmed at autopsy.

A white male student, aged 17 years, was first seen in our office September 25, 1933, complaining of shortness of breath and fatigue. The family history and past history were not significant. He had been in good health until the early summer of 1933, when he first noticed that he tired easily. This fatigue became progressively more noticeable, and he soon developed severe shortness of breath for which he had been hospitalized. X-ray examination at that time revealed a bilateral pneumothorax, with complete collapse of the right lung and the upper one third of the left lung. Symptomatic relief had promptly followed the removal of several hundred cubic centimeters of air from the right pleural cavity.

When seen by us physical examination revealed a rather thin white male, weighing 114 pounds. He was dyspneic on slight exertion, but there was no evidence of cyanosis. He had only a degree of fever, and his pulse rate was only 96. His heart appeared entirely normal. We call attention to this because of later developments. The lung findings were those of a pneumothorax over the upper two thirds of the right lung. Over the left lung there was some increase in the voice and breath sounds and a few scattered small moist rales. Otherwise the physical examination was negative. Laboratory studies of the blood and urine were normal.

Fluoroscopic observation and roentgenograms of the chest showed a rather uniform collapse of the upper portion of the right lung by pneumothorax, about 60 per cent by volume. There was scattered fibrosis in the uncollapsed portion of this lung, and a generalized fibrosis throughout the left lung, with numerous small areas of lessened density, giving the appearance of honeycombing. The absence of calcium was conspicuous. The patient had a right sided pneumothorax which, from the history, was spontaneous and also bilateral. The lesion in the lungs was so unusual that cystic lung disease was considered the probable diagnosis.

In order to give the patient further study he was temporarily kept at bed rest and gradually returned to a relatively normal routine. Because of irregularity of expansion and a tendency to localized ballooning of the lung tissues, small pneumothorax refills were given and the lung permitted to reexpand gradually over a period of several months. During this time the patient was not permitted to return to school but otherwise participated in his usual



activities. Study of lungs following complete re expansion showed an unusual type of fibrosis with honeycombing which we definitely diagnosed as congenital cystic lung disease.

The patient's course continued uneventful for about a year when he noticed a return of symptoms. He reported to the office and was found to have a bilateral spontaneous pneumothorax, with about 90 per cent collapse of the right lung and 30 per cent collapse of the left. Withdrawal of 600 c.c. of air from the right pleural cavity relieved symptoms of respiratory embarrassment, so patient was not hospitalized. The following night he became suddenly worse. When seen at his home soon thereafter, he was found sitting in a chair gasping for his breath. One thousand cubic centimeters of air were removed from the right pleural cavity, which relieved his acute distress; the patient was immediately taken to a hospital. On arrival at the hospital he was cyanotic and again gasping for air. Both pleural cavities were aspirated and a needle retained in the right pleural cavity and connected to a water bottle to permit constant aspiration on this side. Despite this, and repeated aspirations on the left side, dyspnea and cyanosis again returned, and it was necessary to connect the left pleural cavity with a water bottle for continuous aspiration. By continuing constant aspira-

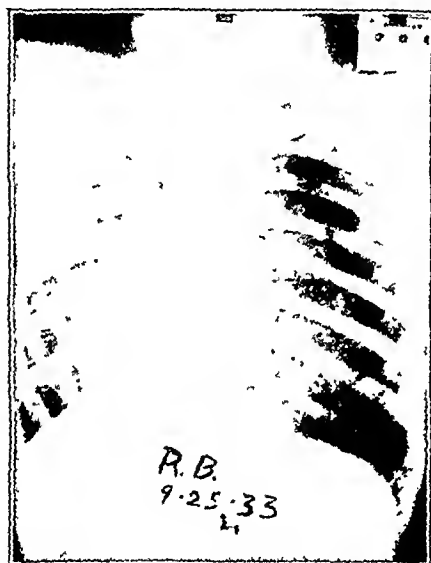


Fig. 1.



Fig. 2.

Fig. 1.—R. B. 9-25-33. Film made when first seen shows pneumothorax on right; generalized fibrosis with multiple honeycombing throughout both lungs.

Fig. 2.—R. B. 4-7-34. Right lung almost completely re-expanded.

tion of both pleural cavities the patient soon became comfortable and made an uneventful recovery. He was discharged from the hospital five weeks after admission, although neither lung had completely re-expanded.

In January, 1935, x-ray showed the right lung had completely re-expanded, but there was a small area of pneumothorax over the left upper lobe. A lung mapping with lipiodol at this time showed the oil filling numerous small dilated areas in the lower portion of both lungs. This finding, coupled with failure to find tubercle bacilli in the sputum after repeated search over a period of one and a half years, confirmed our impression that we were not confronted with tuberculosis; since the patient was now symptom free, he was permitted to return to school.

Patient's course continued uneventful for the next nine months when he developed an acute respiratory infection manifested by cough, expectoration, dyspnea, and fever. Lung findings at this time were those of an acute tracheobronchitis. Examination of the heart revealed an apical systolic murmur and a definite accentuation of the second pulmonic sound.

Electrocardiogram was normal. Although he recovered from this infection, he had recurrences, and his dyspnea became progressively worse. Five months later x-ray examination revealed that both lungs had completely re-expanded and for the first time showed evidence of definite cardiac enlargement.

A few months later, while visiting in another city, the patient had another similar acute respiratory infection and was seen by physicians who insisted that he had tuberculosis. Because of this difference of opinion concerning the diagnosis, the patient was admitted to a tuberculosis sanatorium in September, 1936. While under treatment there, he developed signs of progressive cardiac decompensation from which he died in May, 1937.

Autopsy was limited to contents of thorax. There was a moderate amount of edema of the lower extremities and genitalia. On opening the thorax the visceral and parietal pleurae were densely adherent, so that the parietal pleura had to be removed with the lungs. The diaphragm and pericardium were also adherent to the lungs. On opening the pericardium there was found about 100 c.c. of clear straw-colored fluid.



Fig. 3.

Fig. 3.—R. B. 8-7-34. Simultaneous bilateral spontaneous pneumothorax. Large pneumothorax on left and smaller one on right.



Fig. 4.

Fig. 4.—R. B. 5-2-36. Film made in oblique position. Both lungs completely re-expanded. Cystic areas in midzone in each lung.

**Heart:** The heart appeared to be moderately enlarged, particularly on the right side. It weighed 440 gm. after opening. The epicardium was smooth and glistening, and there was a small amount of fat present in the coronary sulci. The myocardium was dark red in color and showed no evidence of scarring. Thickness of right ventricle was 5 mm. and thickness of left ventricle was 1 cm. The right auricle and ventricle were tremendously dilated. The left auricle and ventricle appeared to be normal, except for a slight dilatation of the left ventricle. The endocardium was thin, smooth, and glistening. The papillary muscles of the right ventricle were greatly thickened. The columnae carneae were also thickened. Those of the left ventricle appeared normal. The chordae tendineae were neither shortened nor thickened. The valves were thin and veil-like and showed no evidence of any disease. The valves appeared competent. The circumference of the valves measured as follows: tricuspid 12.5, pulmonic 7.5, mitral 9, and aortic 6 cm. There appeared to be some slight dilatation of the pulmonary cornice. There were no congenital or other abnormalities of the great vessels.

*Aorta:* The ascending aorta, the arch, and the thoracic portion of the descending aorta appeared normal. They were thin and elastic, and showed no evidence of arteriosclerosis. The coronary vessels appeared normal.

*Lungs:* The visceral and parietal pleurae were densely adherent and appeared greatly scarred. Large cystic areas in the lungs could be seen and felt beneath the pleura. They apparently contained air and no fluid. Both lungs appeared to be overdistended and maintained their shape. The lobes of the lungs also showed dense interlobar adhesions. The lungs were filled with formalin and allowed to fix before further sectioning. After sectioning, the cut surface of the lungs showed both lungs to be greatly involved by a cystic change. These cysts were most numerous and largest at the periphery of the lungs. They varied greatly in size from that of normal vesicles up to cysts 3 cm. in diameter. The walls of these cysts were thin and smooth. They apparently communicated with the bronchi. The normal lung tissue was brownish red in color, presumably due to fixation with formalin. There was no evidence of any infection anywhere in either lung or in the tracheobronchial lymph nodes.

To open the thorax, it was necessary to extend the incision down to the abdomen, and this opportunity for exploring the abdomen was taken. The peritoneal cavity was filled with a clear amber-colored fluid. The liver seemed to be enlarged, but the other organs appeared to be normal as far as could be determined by palpation.

*Microscopic.—Heart:* Left ventricle, including coronary artery and portion of mitral valve, was normal. Right ventricle, including tricuspid valve, showed only very slight scarring of myocardium. *Aorta:* Sections from ascending portion and thoracic descending portion were normal. *Pulmonary Artery:* Moderate proliferation, scarring, and slight round cell infiltration of intima. Media appeared somewhat distorted and edematous. *Lungs:* The alveoli varied in size from normal to large cystic structures. They were lined by normal to greatly thinned out and flattened cells. The transition from small bronchioles to alveoli could be seen. The small bronchioles were lined by very small ciliated columnar epithelial cells, much smaller in size than the cells lining the larger bronchi. These cells became flattened and lost their cilia at the point of transition from bronchiole to alveolus. These small bronchioles were greatly dilated. No definite constrictions could be found. The walls of the alveoli were very thin and delicate. There were many heart failure cells in some of the alveoli. The interlobular connective tissue was edematous and much greater in amount than normal. It was slightly to moderately infiltrated by lymphocytes, with occasional focal accumulations of these cells. The blood vessels and lymphatics were numerous and dilated. Some showed hemorrhages. The larger branches of the pulmonary arteries showed the same changes as in the main branch. The pleura was slightly to greatly thickened and vascularized. It was slightly infiltrated by lymphocytes. *Tracheobronchial Lymph Nodes:* Contained a small amount of anthracotic pigment. There were many large mononuclears containing blood pigment in the sinuses. They were otherwise normal.

*Anatomic Diagnosis:* Congenital cystic disease of the lungs; chronic fibrous pleurisy; hypertrophy and dilatation of the right ventricle; chronic passive congestion of lungs; pericardial effusion; edema of lower extremities and genitalia, ascites.

#### COMMENT

Simultaneous bilateral spontaneous pneumothorax from any cause is an unusual accident and, when resulting from congenital cystic lung disease, it is relatively rare. The case here reported presents several interesting features, many of which are similar to that of the case reported by Oechsli and Miles. The patient remained in good health until he was 17 years of age, when the first spontaneous pneumothorax occurred. He lived for four years after this, and died of congestive heart failure. Following the first occurrence of spontaneous pneumothorax, the lungs completely re-expanded before the second bilateral spontaneous pneumothorax occurred. Following the second occurrence of this accident, the lungs again completely re-expanded, and remained so for several months before death. So far as we have been able to find, this is

the second case on record in which this has happened. There was a striking absence of cough, expectoration, fever and chest pain during the greater part of the time he was under observation and when these symptoms did occur, they were easily accounted for by an acute respiratory infection. Unlike the case of these authors, there were no other congenital abnormalities present.

The successful treatment of spontaneous pneumothorax may require removal of air from the pleural cavity, either by repeated or continuous aspiration. Simple pneumothorax, with a small tear in the lung, may need no treatment or at most one or a few aspirations of air. When the lung tear has closed, the air will be absorbed from the pleural cavity. Larger tears with tension pneumothorax require continuous aspiration with a needle, or rarely with a catheter, inserted into the pleural cavity and connected with a water bottle, such as is used in the drainage of an empyema. The end of the tube should be placed just below the level of the water to permit continuous escape of the air, even though the intrapleural pressure should approach normal. With reasonable aseptic precautions infection of the pleural cavity can be avoided. Although this patient repeatedly had needles inserted through the chest wall and for several days needles were left in place in both pleural cavities, no infection occurred, confirming our impression that the danger of infection from without is negligible.

The diagnosis of congenital cystic lung disease presents little difficulty when large cysts are present. In the type of the disease characterized by multiple small cysts the diagnosis may be confusing as the condition may simulate an infectious process, especially tuberculosis. In this case a positive tuberculin reaction further confused the picture. No evidence of tuberculosis, however, was found at autopsy.

There is no satisfactory treatment for this type of cystic lung disease, and the prognosis is poor. The treatment of this patient was symptomatic and the necessity for maintaining constant aspiration from each pleural cavity indicated that there was no communication between the pleural cavities through the mediastinum.

#### SUMMARY

A case of simultaneous bilateral spontaneous pneumothorax resulting from congenital cystic lung disease is reported with autopsy findings. The patient had a bilateral collapse on two different occasions, and following each the lungs completely re-expanded. The lungs had been completely re-expanded for several months before death occurred. Death was directly the result of congestive heart failure. At autopsy no other congenital abnormalities were encountered.

We wish to express the patient for his first Brown and F B Stafford time of death and to D

L Harrell of Norfolk Va. who treated the notes on this patient to Drs W E under whose care the patient was at the ity Va. who performed the autopsy

#### REFERENCES

- Oechsl, W R, and Miles, S H. Simultaneous Bilateral Spontaneous Pneumothorax, *Am Rev Tuberc* 30: 239, 1934.  
Glickman, L G, and Schlomovitz, B H. Simultaneous Bilateral Spontaneous Pneumothorax Complicating Pneumococcosis, *Am Rev Tuberc* 34: 390, 1936.

# CORRELATION OF HISTOLOGIC STRUCTURE WITH CLINICAL FEATURES\*

## II. THE CASE OF MALIGNANT NEOPLASMS

O. C. GRUNER, M.D.,† MONTREAL, CANADA

THE structure of malignant new growths, which is revealed by the classical histologic methods of study, forms the basis of the prevailing conceptions as to their nature. Modifications of these conceptions become necessary whenever some essential improvement in technique appears. Tissue cultures have revealed unexpected properties in the tumor cells. The wet-film method applied to absolutely fresh tissue has shown that many of the cells in the neoplasm are not rigidly fixed within a particular kind of architecture, and has disclosed objects previously not suspected. Perhaps of all the pictures, that which dark-field illumination provides is the most striking, because the intensely active movement of the particles in the neoplasm is thus for the first time brought to light.

Histologic structure cannot be altogether divorced from physiologic behavior. The phenomena observed during life must go with corresponding cellular elements, so that observation can only have been imperfect if these events do not accord with the clinical phenomena. This view was discussed in connection with goiter‡ where it is very evident that the clinical pathologist's findings are widely at variance with the clinician's observation as to presence or absence of toxic symptoms, etc. It was shown that a more exact analysis of thyroid tissue would bridge the discrepancies. Starting with the thyroid unit, and filling in the variable details, all gradations of disease can be amply provided for.

So, too, with cancer. Only, in the case of malignant neoplasms, we distinguish two series of components, one corresponding to the parenchyma of organs, and the other consisting of superadded elements not ordinarily found in noninfective diseases. There is an essential dualism in the structure.

Disregarding for the moment the stereotyped classification of tumors, the first step in the analysis is to catalogue the various elements which ever occur in tumor tissue. The next step is to assess the relative degrees of importance of each element and determine its chronologic relation to every other. From these data the essential features of each case can be put together into a simple formula—following the method proposed in the paper on goiter. Finally, the various formulas are compared, analyzed, and correlated with the clinical

\*The researches upon which this paper is based were carried out under a grant for cancer research contributed to Dr. Edward Archibald by an anonymous donor.

†Research fellow, McGill University, Montreal.

‡The previous article appeared in the J. LAB. & CLIN. MED. 23: 431, 1938.

Received for publication, March 23, 1938.

data, with the ultimate object of seeking the explanation of the various features of the disease in the given patient and the nature of malignancy in general.

### HISTOLOGIC CANCER UNIT

The histologic unit of cancer tissue comprises cells and a matrix carrying vascular channels and nerve filaments. The cellular components may be grouped into (1) fixed, (2) free-floating tumor cells, and (3) wandering cells (histiocytes).

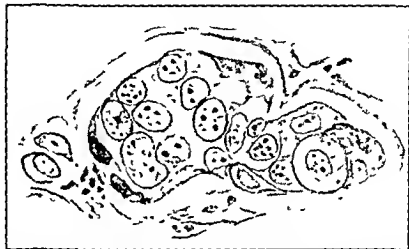


Fig. 1.—Carcinoma solidum of breast. Growing point of one of the cell columns. Slightly schematic drawing to show the dusky trophic cells and the paler-staining "essential" cells. This depicts the "cancer unit" (oil immersion, Oc. 15).

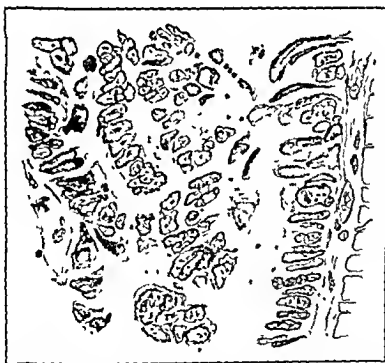


Fig. 2.—Adenocarcinoma of colon. Slightly schematic drawing of a portion of an acinus (cancer unit) to show the dusky trophic cells and paler-staining "essential" cells. Many free cells are shown, and some chromidial granules (oil immersion, Oc. 15).

1. *Fixed cells* vary in shape in the different classes of neoplasm (squamous, spheroidal, and columnar-celled, etc.), and in each case are of two kinds: (a) the trophic cells or trophocytes, which have a dusky cytoplasm, and a relatively large pachychromatic nucleus; (b) the essential or proper cells, which are larger, show a pale-staining cytoplasm, and a relatively smaller leptochromatic nucleus. (Figs. 1 and 2.) This division is general in both animal and vegetal organs. Thus, in normal histology, the Sertoli cells of the testis, the basal cells of the epithelium, the argentaffine cells of the neuro-

endocrine systems are trophic cells, and the germ cells, epithelial cells, and ganglionic cells are the respectively corresponding essential cells. During youth, food material normally flows from trophocyte to essential cell; in senescence the flow is in the opposite direction, according to Borrel. In addition to these fixed cells, many malignant neoplasms also show giant cells with bizarre nuclei; these may, however, also be of the nature of trophocytes.

2. *Free-Floating Cells*.—These comprise (a) small and large "naked" nuclei; (b) small triangular cells like miniature plasma cells; (c) foamy cells, nuclei; (d) small triangular cells like miniature plasma cells; (e) foamy cells,

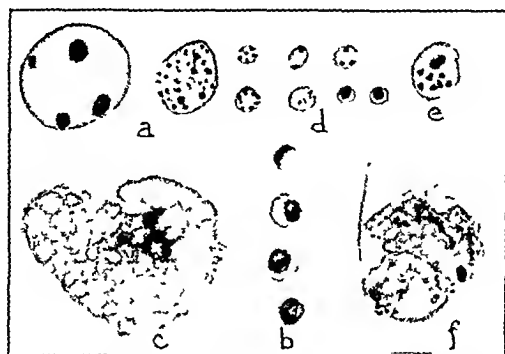


Fig. 3.—Free-swimming cells: a, a', large and small "naked" nuclei; b, cells like plasma cells; c, foamy cell. Chromidia: d, type "Ib" and "Ic," e, type "Ie," "octet." Filamentous forms: f, noded lines in cancer cell nucleus and cytoplasm (Zeiss, oil immersion, Oc. 15).

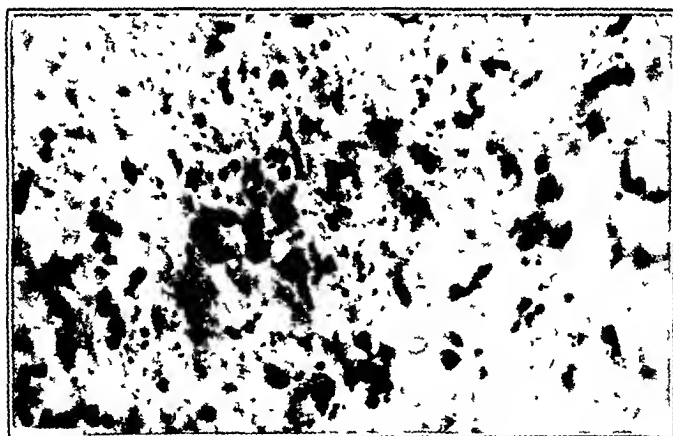


Fig. 4.—Sarcoma. (a) To show the granulations of types Ib and Ic (small and large granules). The latter have a narrow halo. (Zeiss lens, obj. 20, Oc. 15.) (b) Another preparation showing larger granulations among the tumor cells.

some full of fat droplets and amoeba-like in form, others containing other granules and no fat; (d) tailed cells (Fig. 3).

These cells can only be identified in wet film or in vital stained preparations. In other words, the operation material must be subjected to cytologic analysis within five minutes of removal from the body, if all these cell forms are to be demonstrated. Smear preparations made like blood smears (with or without dilution in isotonic saline) show them quite well, and dark-field study is advisable on other portions of material as soon as the films, etc., have

been safely deposited in the fixing fluids (The wet film method<sup>1</sup> consists in plunging the still wet tissue scrapings into Schaudinn, or Bouin, for subsequent staining by Mayer's hemalum or by iron hematoxylin—the most informative nuclear stain)

(a) *Naked nuclei* These vary much in size. Their outline is circular or oval or indented on both sides. Some have coarse granules of chromatin, with clear intervening material, and resemble the embryonic forms of some protozoan cells of stagnant water. Others show flake or tigroid markings, with a color reaction to Leishman, suggestive of them being dying cells, whose cytoplasm has been reduced from a mere halo to the vanishing point. The small germ center cells of lymph nodes (lymphoidocytes) often present the same appearance in film preparations of lymphoid tissue. Nucleolar matter is present. Some of these cells may appear as round shells enclosing a bundle of pyriform bodies. In the dark field, some of these cells have a gregarine like appearance, and alter their shape continually, indicative of irritability.

(b) *Miniature plasma cell like forms* These are triangular, the nucleus being round, trachychromatic and pachychromatic, and forming the bulk of the cells, while the cytoplasm is dusky. They are seen best of all by mingling the fresh scraping with the methyl green pyronine stain of Pappenheim, and immediately covering the mixture with a cover slip.<sup>2</sup> After a short time the different cells become clearly distinguishable.

(c) *Foamy cells* The most striking are found in breast tumors and show protoplasmic masses full of relatively large fat globules (which also occur free throughout such films), and show one or two quite small nuclei. Others appear foamy in virtue of being packed with refractile granules, but differ in failing to give a fat reaction.

(d) *Tailed cells* These were known to the earliest histologists (They figure in L. S. Beale's plates published in 1870). Some of them are of very large size and contain inclusion bodies—either scattered (Fig. 7) or in aggregated masses like included protozoa. The latter would be described as the normal segregation apparatus of the cell by some cytologists.

In passing, it may be noted that these various free floating cells are all referred to in J. Koch's monograph<sup>2</sup> as successive stages in the development of a protozoan parasite, the *cellula cancerosa*, in other words, he regards them as foreign cells and not host cells at all. Other observers in recent years have come to realize that organisms can pass from ultramicroscopic dimensions through all gradations up to the size of the largest body cells.

3 *Wandering cells* include macrophages, monocytes, histiocytes, and various inflammatory cells. The monocytes are a very striking feature of cancer tissue cultures, moving as they do among and all over the tumor cells. In some cases, in fact, the tumor appears actually to be composed of macrophages. In histologic sections, however, such cells may escape notice or are only recognized by their nuclear form—the cytoplasm being relatively inconspicuous. The nuclei are liable to be mistaken for the granulations presently to be discussed, and when the macrophages are active, the engulfed objects may be



mistaken for specific inclusions. The number and varieties of the inflammatory cells in general depend on the concurrent secondary infection in the neoplasm, but the cell formula is worthy of record as part of the histologic analysis of a given laboratory specimen.

These macrophages are, of course, not specific for cancer, but the fact that they are much more plentiful in malignant neoplasms than in septic, tuberculous, and other tissues gives them a quasi-specificity.

#### CHROMIDIAL GRANULATIONS

To this point the cancer unit has been described as to its cellular elements. A second series of components has now to be discussed—the chromidial granulations. These are so named in the present study because they are defined as having nuclear or chromatinic composition, to distinguish them from other granules which are certainly cytoplasmic and nonspecific. Those chromidia pervade the tissue. They are intermediate between the histologic cancer unit and the actual unit.

Table I includes a list of these components of the cancer unit.

TABLE I

#### THREE GROUPS OF CHROMIDIAL GRANULES AND INCLUSION BODIES

- I. *Granular forms.* These occur in the intercellular tissue and in the connective tissue spaces as well as within tumor cells.
  - (a) Submicroscopic: visible by dark-field method (elementary bodies).
  - (b) Microgranules: up to  $1\mu$  in diameter. As these often show a halo, they constitute the smallest "inclusion bodies."
  - (c) Macrogranules: (from 1 to  $3\mu$ ).
  - (d) Both (b) and (c) in the same tissue (Figs. 3, 4).
  - (e) Packets of (usually) eight particles, sometimes found within an oval deeply oxyphile sharply contoured "cell." These bodies are both trachy- and pachychromatic (Fig. 3).
- II. *Cell inclusions in the usual sense.*
  - (a) Lying within signet-ring cells. The classical Plimmer bodies belong here. Blastomycetes have a similar appearance (Figs. 5, 6).
  - (b) Smaller bodies, resembling coccidia.
  - (c) Metachromatic masses within tumor cells, suggestive of plasmodial bodies; therefore, resembling protozoa.
  - (d) Pachychromatic oval bodies resembling torulae or yeasts.
- III. *Filamentous forms.* These are (a) noded, (b) spirillar or wavy in form. They are located in mononuclear leucocytes, in the circulating blood, and also occur in tumor cells—usually in the cytoplasm, sometimes in the nucleus. When multiple and intertwined, as may occur in some cases of advanced carcinoma, they resemble a small mycelium.

All these bodies are stainable with dyes, but all are not visible in histologic preparations. Some of them (I-a) are only recognizable by means of dark-field illumination or by means of the Zeiss polychromar, and only in the freshly obtained tumor juice or "milk." These include innumerable extremely minute granulations in a state of highly active movement. Others are only seen in the fresh blood from a cancer patient, though with difficulty because of their only momentarily coming into view from the depths of the specimen; the

larger bodies present distinctive (vermicular or undulating) movements. Others are only seen with certainty within the blood cells (monocytes) in Leishman preparations.

*The Shape of Granulations in Stained Preparations.*—Those of the smallest size are spherical. Even the smallest may be enclosed in a clear halo like the cell inclusions in various virus diseases. Other forms are dumbbells, "haltères," oval forms with lateral buds, pyriform shapes, beaded forms, irregular forms, and filamentous forms. Some of these are exactly like wandering leucocytes seen among the epidermal cells of an epithelioma; some resemble yeasts. Mulberry forms suggest autogenous multiplication. Bizarre shapes, indicative of



Fig. 5.—Free-swimming tumor cell with contained Plimmer body. Vital staining with neutral red.

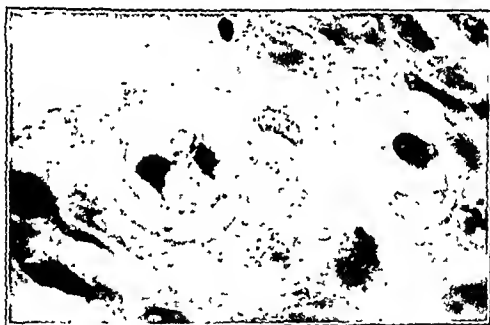


Fig. 6.—Large carcinoma cell containing Plimmer body in a state of division with free chromosomal granules.

pseudopods, suggest livingness (fresh preparations). Irregularly quadrilateral shapes in the wet specimen, with refractile granules in them, exhibit motility. Octets whose components are pyriform suggest a reproductive form (ascospores), though they are generally passed over as caryorrhectic and pycnotic leucocytes, or as phagocytized matter. Forms usually described as mitochondria may occur, among which are filamentous forms marked with nodal thickenings. (These can also be demonstrated in blood cultures.) The larger inclusion bodies, up to the size of Plimmer bodies, are also spherical, and usually present a halo.

*Plimmer bodies* are oval, vesicular, or rounded, sometimes hyaline bodies within large cancer cells, the host-cell nucleus being the same size as the in-

clusion, but usually of crescentic shape. Within the body occurs a spherical deeply staining mass of relatively small size. Fig. 5 shows one of these bodies in a wet preparation, vitally stained with neutral red. The presence of definite nuclear structure is demonstrated in Fig. 6, and shows that these bodies are not degenerative products.

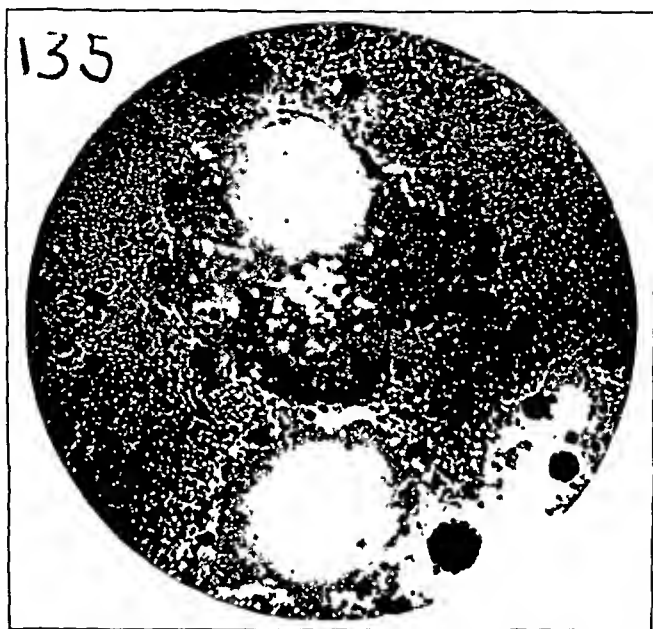


Fig. 7.—Large carcinoma cell containing numerous "virus bodies" or macrogranules. Smear of fresh operation material. Leishman stain. The background is stippled with microgranules of varying size, which include also elementary bodies only seen with dark-field observation.

#### Lumen of Bowel

18	24		
0			
38	1240	1	
1			
56	289	6	
4			
48	430	10	
2			
88	522	24	
6			
90	477	19	
11			
68	530	74	
13			
95	391	95	
17			
112	249	148	
22			

Advancing Edge  
No Tumor Cells

Fig. 8.—Edge of an adenocarcinomatous ulcer. Chromidial analysis of the tissue. Each horizontal line marks off a high-power field of the section. The horizontal lengths are proportional to the number of fields occupied by tumor tissue at the respective levels.

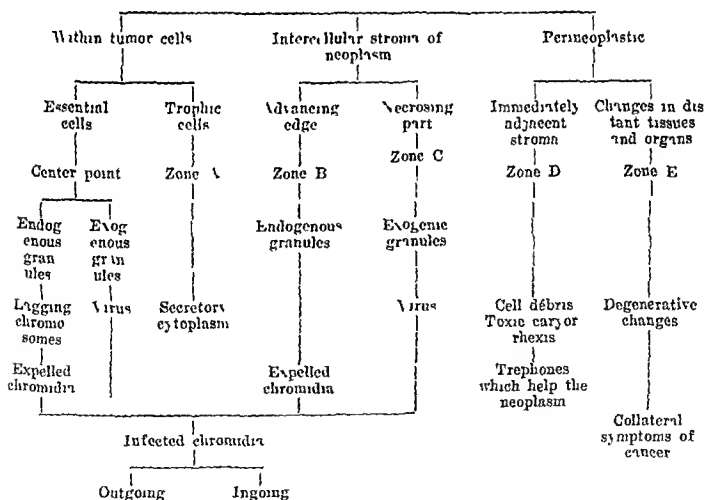
Numerals in the rectangles: left top corner is the number of microgranules, left lower corner is the number of signet-ring cells. Right hand lower corner gives the number of hyperchromic nuclei. The large number in the middle of each rectangle gives the number of macrogranules.

## CORRELATION OF CELLS WITH GRANULATIONS

Having listed all the elements which can be found in tumor tissue of whatever kind, we proceed to place them in position. First, a plan of the tissue is drawn up to show a low power magnification and thus assign an orientation—division into districts according to histoanatomic structure and to the position of the several cell types. The several kinds of chromidia are now placed, and the pattern formed is thus made evident. They show focal groupings, zonal groupings, or diffuse scattering. Some are intracellular, either in the resting or in the dividing cell, others are intercellular. Some of the latter are between the tumor cells themselves, others are in the necrotic portions or in the duct lumina.

Thus, Fig. 8 shows the actual numbers of granules of various kinds in the plane of one section as met with in successive histoanatomic regions. This correlation is more clearly brought out in the accompanying schema, which assigns a particular place and function to the chromidia.

TABLE II  
HISTOLOGIC RELATIONSHIPS OF CHROMIDIAL GRANULES



The tumor cells are not only static, they are also dynamic, and, therefore, a place has to be found in the scheme for the occurrence of mitosis, especially as one would naturally expect chromidia to show a relation to cell division, especially when mitosis is of pathologic form. They are, therefore, looked for in mitosing cells—whether normal or pathologic. The most important study of pathologic mitosis is that recorded by Poltzer,<sup>3</sup> that of the effect of x rays

on the cells. The fact that chromatolysis occurs under such conditions, is the argument for saying that the chromidia are merely and solely degeneration products.

The chief importance of correlating chromidialization with mitosis is that it provides a point of time from which to measure the events in the tissue. For, if the various chromidial forms are chronologically related, it is necessary also to ascertain how they fit in with the changes continuously proceeding within the tumor cells. Just as the chromidia culminate in a particular type so the tumor cells culminate in a particular form of structure. If the chromidia favor dividing cells, it is clear that every cell of a neoplasm is not equally "malignant," meaning by malignancy "ability to harbor and nourish virus." That is, only virus-containing cells are malignant at any given time.

Hence, in assigning a dual nature to malignancy, the power of multiplication cannot be restricted to the tumor cells alone. The chromidia evidently multiply also, and though they are apparently fixed or stable objects, without biologic organization, their varying sizes may correspond with differences of age. As with all biologic entities, a cycle of development is conceivable, the most minute granules being at one end of the scale and the Plimmer bodies at the other. The various bodies listed in Table I provide a means of "typing" cancerous tissue, and a number can be assigned to each case according to the kind of body which appears furthest on in the list. Thus, when "octets" are found, even in small numbers, the type would be recorded as "Ie," rather than "Ib," "Ic," or "Id"; and if inclusions in signet-ring cells are present, the type is called "II."

The successive types represent a progression toward maturation of the cancerous process, though every neoplasm is not inevitably going through the whole series. Further, the chromidia in some specimens of cancer suggest that an alternation of forms may occur, and this implies the possibility of some of the bodies being dioecious (or multisexual) in nature. (Thus, octets would correspond to ascosporeangia.)

But the cycle applies to cancer only in a collective sense, because it only emerges from an analysis of a succession of cases, and not from repeated

TABLE III

ANALYSIS OF CASES ACCORDING TO SITE OF PRIMARY, SHOWING THE FREQUENCY OF OCCURRENCE OF THE VARIOUS GRANULE TYPES

	TYPE I					TYPE II				TYPE III		TOTAL
	a	b	c	d	e	a	b	c	d	a	b	
External and squamous carcinoma (face, mouth, throat, etc.)	2	3	7	7	1	9	1	11		18	1	59
Uterine epithelioma		7	6	9		7		2	2	25	14	17
Gastric		1	6	4	2	8		1	1	13	3	39
Intestinal	2	5	6	1	6	13		1	6	7	8	51
Breast	7	4	9	7	2	30	1	7	1	10		76
Sarcoma	1	4	1	2		3		2		4	3	19
Lung		2		2	1	2				8	6	20
Unclassified sites		2	1			4				10	1	13
Total	12	28	36	32	12	76	2	24	10	95	36	349

biopsies of single cases. In the series analyzed in Table III, the II type, for instance, is rare in sarcoma and some forms of carcinoma.

To conclude, the complete interpretation of the cancerous tissue entails superposing the several phases of chromidia upon the successive histologic loci. The abundance or paucity of the various types is equally important in forming an opinion as to the degree or severity of the disease and the biologic stage to which it has reached.

#### DISCUSSION

The evidence available in favor of the suggested method of interpreting the histologic structure of neoplasms can only be referred to briefly within the limits of this paper.

Reduced to strict essentials, the granulations may be (1) artifacts or adventitious matter, (2) normal cell structures or cell products, (3) pathologic cell products and by products, that is, degenerative in nature, pathologic mitoses, (4) bacterial contaminations or secondary infections. The need to exclude the first item is most evident when studying material by dark ground illumination. The second and fourth items are not difficult to eliminate. Hence the real problem is the third—whether the chromidial granules are simply degenerative in origin and not causally related to the disease. The arguments customarily raised against a causal relationship may be passed over, because they are repeatedly and unfailingly brought forward in the literature. There are nevertheless very good reasons for advocating an opposite conclusion. Among them are the following:

*Histologic.* (1) The fact that host cells and cell fragments can be mimicked by foreign cells and organisms. Recognition of this possibility underlies the repeated efforts to devise differential staining methods (with or without optical devices) for distinguishing with certainty two otherwise exactly similar objects. (2) The demonstration that if a dilute culture of cryptomyces is admixed with a fresh preparation of normal blood, it can be very difficult to discover the presence of the organism, similarly, that a histologic section of ascomycete peritheciium can present a very close resemblance to the tissue of round celled sarcoma within restricted microscopic fields. (3) As a practical fact, eminent pathologists insist that even a cancer cell is indistinguishable from an ordinary body cell (this being an argument that the cancer cell is necessarily always a host cell). (4) Appearances alone will not rule out the possibility of some chromidia in a cell being infective. (5) The "octets" of Table I—eight sharply defined refractile bodies of oval or pyriform shape—are not a feature of the ordinary course of leucocyte erythrohexis. (6) Divisions and mitoses can be seen in the signet-ring cell inclusions. (7) Experimental inoculations with cultures of cryptomyces produce bodies exactly similar to those under discussion, and in this case the "chromidia" are definitely identical with phases of the injected organisms. (8) Diagnosis that a biopsy is cancerous solely on the basis of the presence of these bodies has been proved correct by the subsequent history, when the disease finally became clinically and histologically established.

*Clinical:* (1) Tumors showing "degeneration" are more malignant than those which retain well-formed structure throughout; in other words, the more numerous the granulations, the more rapid the spread of the disease. (2) The evidence of Table III, though this needs supplementing by an exact analysis of the after history in every case therein, in order to show the higher types as clinically more malignant or associated with a wider dissemination through the body. Actually, however, this correlation is not uniform, being affected partly by the patient's reaction and partly by the previous medical history. As was discussed in regard to goiter, several diverse factors contribute to the picture. (3) Correlation of blood picture with chromidial types. Discrepancies are found to occur here because of the effect of concurrent treatment. The inclusion bodies disappear completely under radium therapy, remaining absent for a long period of time; x-ray therapy has a similar effect, but of

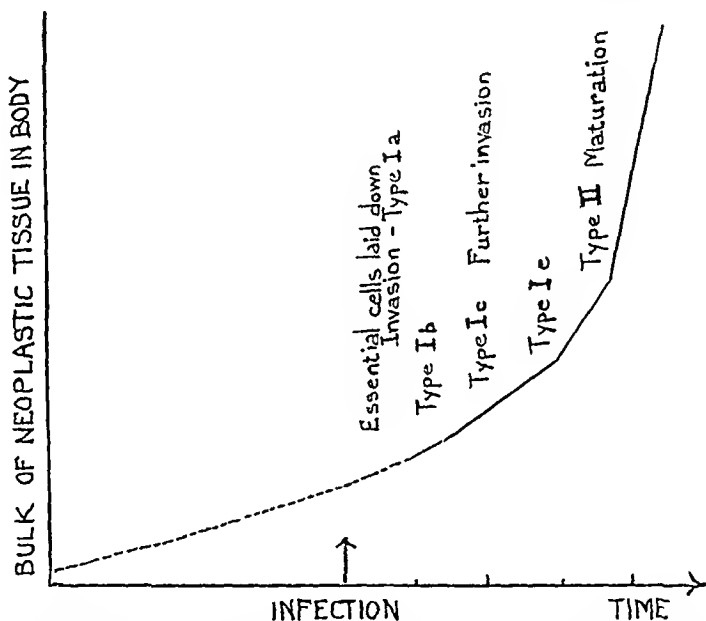


Fig. 9.—Relation between rate of progress of cancer and type of chromidialization. The rise in the curve represents schematically the increasing bulk of neoplastic tissue in the body.

only a few weeks' duration. (4) Biochemical data: determinations of the pH of the blood, of blood calcium, and of glucose tolerance do not give uniform results in all kinds of cancer. This can be explained on the basis of the histologic factors. Thus, dark-field study of fresh tissue shows abundance of fatty acid (anisotropic crystals) and the presence of crystals of calcium oxalate in some tumors and not in others. (It is suggestive that both these findings are also a feature of fungal cultures.) Neoplasms may also be described as being sometimes open, sometimes closed, according to the degree of fibrosis, or blocking of vascular channels by columns of tumor cells, or abundance of chromidial formation. Certainly the blood picture shows abeyance of the distinctive changes ("quiescence") when the tissue also suggests a "closed" form. (5) The time factor. This presents three relations: (a) the stage of

the disease, (b) the duration of the successive chromidial phases, (c) the incidence of the important precancerous disease (i.e., chronologically, not etiologically precancerous). In some cases the clinical history shows a succession of periods with which the chromidial types also correspond (Fig. 9). Blood studies bring this relation out best, because it is rarely possible to observe the tissue at different periods in one case. The time curve is relative, not absolute; the lengths of the abscissae on the chart bear proportional values only. As to (c), the previous medical history, the date and duration of each important illness prior to the first signs of the neoplasm are recorded in order to mark out the intervals (in weeks or months) between each pair of events. The interval between the first sign of neoplasm and the first recurrence has a definite value (Douglas Webster<sup>4</sup>). These intervals are explained by the histologic structure, as the chromidia also take time for their evolution, even if they only pertain to the constitutional factor underlying the disease. (6) The relation to choice of treatment. The ultimate fact about treatment is the choice between surgery or radiation, or both, which in itself tacitly admits the disease to be local at the onset. Up to a certain point, the local lesion or "primary" is the whole disease, and if it could be completely removed, the case would be definitely "cured." The unwillingness to speak of a cure is the permanent doubt whether removal was complete. The present research points to the conclusion that by the time the tumor is there, the blood already shows the characteristic evidences of virus infection. Hence it is relatively unimportant whether the neoplasm is to be regarded as the factory of the virus (i.e., primarily a local disease to start with) or only its storehouse or granary (i.e., a constitutional disease with local manifestation). In either case local treatment is not alone sufficient to ensure a lasting cure, and cases occur in which local radiation forces the chromidia further on, or into the blood and internal organs. In such cases, the inclusion bodies in the blood are found still present, despite the absence of any other evidence of the disease. This supports the argument that cancer is not just "neoplasm," but a disease requiring general treatment as well.

#### SUMMARY

A method of interpretation is presented which furnishes a means of correlating the structure of neoplasms with their behavior and with the clinical features of the cancer disease.

The essential basis of the interpretation is that there is a dualism of nature in the neoplasms—one component being the cellular one, and the other the chromidial granules. The tumor cells are partly a reaction to virus infection, partly the site of proliferation of the virus, so that only the infected cells are the really malignant ones.

The chromidial granules are not considered to be degeneration products, but inherent in the propagation of the virus. They are thus raised to a position of special significance.



A special analysis of cases studied according to the types of granulation is presented, the associated researches forming the ultimate basis of the present thesis.

## REFERENCES

1. Dudgeon, L. S., and Patrick, C. V.: A New Method for the Rapid Microscopical Diagnosis of Tumours, With an Account of 200 Cases So Examined, Brit. J. Surg. 15: 250, 1927.
2. Koch, J.: Ueber die Ursache und Entstehung des Krebses, Jena, 1936, Gustav Fischer.
3. Politzer, G.: Pathologie der Mitose. Protoplasma Monographien, Berlin, 1934, Gebrüder Borntraeger.
4. Webster, Douglas: Periodicity of Recurrences in Cancer, Brit. M. J. Pt. 1: 41, 1938.

## TOXICITY, THERAPEUTIC ACTIVITY, AND MODE OF ACTION OF SULFANILAMIDE IN EXPERIMENTAL STREPTOCOCCUS INFECTIONS OF RABBITS\*

JOHN A. KOLMER, M.D., HERMAN BROWN, B.S., AND ANNA M. RULE,  
PHILADELPHIA, PA.

WITH THE ASSISTANCE OF MARY F. WERNER

SINCE septicemia of human beings due to beta hemolytic streptococci is usually the result of a rapid overflow of organisms into the blood from a primary focus of infection of the fixed tissues which cannot be effectively balanced by the clearing mechanism, we have employed rabbits in this investigation inoculated intradermally and intravenously with *virulent* streptococcus of this type, with the production of severe local skin lesions, septicemia, and secondary suppurative arthritis, according to the method of Kolmer and Rule.<sup>1</sup> These lesions not only permit direct clinical observations but likewise and more importantly, bacteriologic examinations of the skin, blood, and joints as a means for ascertaining disinfection of these tissues by chemical agents, and in these respects constituting a more satisfactory experimental disease than experimental streptococcus infections of mice so commonly employed.

In a previous communication by Kolmer, Brown, Raiziss, Rule, and Clemence,<sup>2</sup> it was reported that prontosil soluble, while exerting some curative effects upon the local skin lesions, failed to produce complete disinfection in the dose employed (0.015 gm. per kg. daily for five days in succession), being in both respects less effective than pyridyl sulfide in similar dosage per body weight. Both compounds also exerted some bactericidal effects upon the associated septicemia, but were practically ineffective in the treatment of the secondary suppurative arthritis in the doses employed.

The present investigation was confined entirely to a study of sulfanilamide† in the treatment of these experimental lesions, since its lower toxicity has permitted the administration of much larger doses. Furthermore, it has been pos-

\*From the Research Institute of Cutaneous Medicine, Philadelphia.

Received for publication, March 23, 1933.

†Kindly prepared and furnished by the Dermatological Research Laboratories, Philadelphia.

sible to determine quantitatively the concentration of free and conjugated sulfanilamide in the blood in relation to toxicity and therapeutic effectiveness, as well as of the compound in the local skin lesions in relation to disinfection, as determined by clinical observations and bacteriologic examinations.

Furthermore, while the maximum tolerated and minimal lethal doses of sulfanilamide and its derivatives for the lower animals per kilogram of weight by different routes of administration have been thoroughly studied by various investigators, its chemopathologic effects have not been reported upon, so that we have included this important phase of the subject in the present investigation.

#### TOXICITY OF SULFANILAMIDE IN RELATION TO CONCENTRATION IN THE BLOOD OF NORMAL AND NEPHRITIC RABBITS

With the particular brand of sulfanilamide employed Raiziss, Seviat, and Moetsch<sup>3</sup> have reported the maximum single tolerated dose for mice by subcutaneous injection to be about 2 gm per kg of weight. By oral administration to rabbits the maximum single tolerated dose was about 15 gm per kg, since 94 per cent of animals survived the administration of this amount.

In our experiments rabbits have lived indefinitely following ten daily subcutaneous injections of 0.004 to as much as 0.160 gm per kg (corresponding to 96 gm per 60 kg), each dose being divided into two portions, given six hours apart.

By oral administration the maximum tolerated dose was between 0.2 and 1.0 gm per kg daily, divided into two doses six hours apart, as an animal survived after six daily doses of the latter, a third animal succumbing after three daily doses of 2.0 gm per kg.

Quantitative analyses of the blood for free sulfanilamide by the method of Marshall, Emerson, and Cutting<sup>4</sup> were made about twenty-four hours after each daily administration, and because of rapid absorption and elimination, showed none of the compound in the blood for the first one to three days. After this time, however, the compound accumulated faster than it could be eliminated, but even a daily dose of 0.160 gm per kg by subcutaneous injection (divided into two doses six hours apart) did not result in a concentration of free sulfanilamide over 1.4 mg per 100 cc (ninth day). A daily dose of 0.2 gm per kg by oral administration (divided into two doses six hours apart) resulted in a maximum concentration of 0.8 mg per 100 cc (on the ninth day), but a daily dose of 1.0 gm per kg by oral administration (divided into two doses six hours apart) produced a concentration of 12.5 mg per 100 cc on the seventh day when this animal succumbed from the toxic effects.

In a second experiment the compound was given orally to four rabbits in a daily total dose of 0.2 to 0.8 gm per kg (divided into two doses daily) for five days. One receiving 0.4 gm per kg twice daily died after the eighth dose (fourth day), indicating that about 0.3 gm twice a day by oral administration was about the maximum tolerated dose by this route and manner of administration.

Free and conjugated sulfanilamide (para acetylamino benzenesulfonamide) was determined in the blood of each animal three hours after the first and

seventeen hours after the second dose each day by the method of Marshall.<sup>5</sup> Because of rapid absorption, maximum amounts were found in the blood as early as three hours after the first dose in each animal. It was noted that two daily doses of 0.1 and 0.2 gm. per kg. by oral administration did not result in a concentration of free sulfanilamide higher than 6.5 mg. per 100 c.c. of blood, whereas it required the maximum tolerated dose of 0.3 gm. per kg. twice daily before a concentration of 10 to 14 mg. per 100 c.c. resulted. This is believed to be the concentration required for pronounced therapeutic effects. A twice daily dose of 0.4 gm. per kg., which was lethal on the fourth day, resulted in a maximum concentration of 10.6 mg. per 100 c.c. three hours after the fifth dose (third day), the animal succumbing after the eighth dose (fourth day) with 6.5 mg. per 100 c.c.

The blood concentration of the conjugated or acetyl compound, the production of which may be a defensive mechanism of detoxification, varied from 1.4 mg. per 100 c.c. in the case of a rabbit receiving 0.1 gm. per kg. twice daily to as high as 29 mg. in the case of a rabbit receiving 0.4 gm. per kg. twice daily, but the therapeutic effectiveness of this conjugated compound is still uncertain.\* If it contributes to therapeutic effectiveness, the total sulfanilamide in the blood of rabbits has varied from 10 mg. per 100 c.c. in the case of 0.1 gm. per kg. twice daily to as much as 37 mg. when 0.4 gm. per kg. is given twice daily by oral administration.

In a third experiment six rabbits were given an intravenous injection of mercuraphen in dose of 0.002 gm. per kg. for the purpose of producing a mercurial nephritis.† Four days later the blood urea nitrogen varied from 60 to 80 mg. per 100 c.c., indicating well-marked nitrogen retention from the kidney injury. Two controls died of this nephritis in thirteen to fifteen days.

The four remaining animals were given sulfanilamide twice daily in dose of 0.1 to 0.4 gm. per kg. by oral administration, beginning three days after the injection of mercuraphen. Each daily dose was divided in two, given six hours apart.

Because of the nephritis, the maximum tolerated dose was reduced from 0.3 to 0.2 gm. twice daily per kg., presumably because of interference with the elimination of the compound. In confirmation of the observations of Marshall and his colleagues,<sup>4</sup> it was observed that the nephritis resulted in the accumulation of both free and conjugated sulfanilamide in marked degree as compared with normal rabbits; also that toxic effects were produced by a smaller amount of free sulfanilamide in the blood of nephritic than in normal rabbits and indicating the need for extra caution in dosage for human beings with nephritis. For example, the dose of 0.1 gm. per kg. twice daily resulted in a maximum concentration of 5.5 mg. of free and 4.5 mg. of conjugated sulfanilamide per 100 c.c. of blood in the case of a normal rabbit, but of 9.2 mg. of free and 21.2 mg. of conjugated in the case of a nephritic rabbit; a dose of 0.4 gm. per kg. twice daily resulted in a maximum concentration of 12 mg. of free and 29 mg. of conjugated

\*Dr. George W. Raiziss informs us (personal communication) that he believes it to be therapeutically effective in streptococcus septicemia of mice.

†Histologic examination has shown the production of severe tubular nephritis (nephrosis) characterized by degenerative and desquamative changes in the tubular epithelium with calcareous infiltration.

sulfanilamide per 100 c c of blood in the case of a normal rabbit, but of 22 mg of free and 38 mg of conjugated in a nephritic rabbit

In the normal rabbit it was found that when a twenty four hour level of free sulfanilamide, amounting to 10 mg per 100 c c, was maintained for a few days, death ensued. This level was only attained when the enormous dose of 0.4 gm per kg was administered twice daily and then only after elimination had begun to be impaired. In other words, it would appear that attempts to attain a blood level of about 10 mg per 100 c c during clinical treatment may be hazardous for human beings unless they have a greater tolerance for sulfanilamide than rabbits. Attempts to reach such levels may account for the appearance of the toxic effects reported in the literature.

In an additional experiment the blood concentration of free and conjugated sulfanilamide was determined in a normal and a nephritic rabbit receiving a single dose of 0.3 gm sulfanilamide per day per kilogram of weight. The latter showed a blood urea nitrogen of 54 mg per 100 c c three days after the injection of mercuraphen, when the administration of sulfanilamide was begun.

It was observed that there was a very rapid absorption of sulfanilamide from the gastrointestinal tract, about 50 per cent of the total free sulfanilamide being found in the blood in the first half hour. This applied to both the normal and nephritic animals and in both the maximum amounts of free sulfanilamide were found in from two to three hours. In the normal rabbit there was a decrease in free sulfanilamide to negligible amounts in thirty two hours, while the nephritic rabbit showed a somewhat delayed elimination. In thirty two hours there was still 2.5 mg free sulfanilamide as compared to 0.9 mg per 100 c c in the normal animal.

This difference was much more definite when the curves of conjugated sulfanilamide were considered. Here it was found that a considerable accumulation of the conjugated compound occurred in the nephritic rabbit, equal in amount to the free sulfanilamide, although the peak of this curve was delayed several hours over that of the free sulfanilamide. The conjugated sulfanilamide found in the normal rabbit reached a maximum value somewhat earlier and was only about one half that of the free sulfanilamide. Little or no conjugated sulfanilamide was detectable in the first half hour, although, as has been indicated, considerable free sulfanilamide already appeared in the blood at this time.

These differences point to an interesting speculation as to the mechanism of *in vivo* conjugation. Combined sulfanilamide must be formed either in the gastrointestinal tract and be absorbed and eliminated more slowly, or it is actually conjugated during passage through the fixed tissues. Retarded elimination is indicated in both the normal and nephritic animal, more so in the latter since general elimination is definitely impaired. Slower absorption of conjugated than of free sulfanilamide may account for the delayed appearance of the former if it is formed in the gastrointestinal tract, but such a mechanism does not account for the greatly increased conjugated sulfanilamide in the nephritic rabbit. This might be due to a selective kidney elimination, but defective elimination should also bring about a great increase of free sulfanilamide.

TABLE I  
EFFECT OF SULFANILAMIDE UPON LOCAL STREPTOCOCCUS SKIN LESIONS OF RABBITS\*

NO.	DAILY DOSE PER KG.** (GM.)	ROUTE OF ADMIN.	FIRST DAY BEFORE TREATMENT		SECOND DAY		THIRD DAY		FOURTH DAY		FIFTH DAY		SEVENTH DAY		TENTH DAY	
			CLINICAL	CULTURE	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.
1	0.004	Subcut.	4	+	4	+	2	+	1	+	Absc.	+	Absc.	+	Absc.	+
2	0.008	Subcut.	4	+	4	+	2	+	1	+	1	+	Absc.	+	Died	+
3	0.08	Subcut.	4	+	4	+	2	+	1	+	1	+	Absc.	+	Absc.	+
4	0.2	Orally	4	+	4	+	3	+	1	+	1	+	Absc.	+	Absc.	+
5	0.5	Orally	4	+	4	+	3	+	3	+	3	+	Absc.	+	Absc.	+
6	1.0	Orally	4	+	4	+	3	+	Died	+	4	+	Absc.	+	Absc.	+
7	Control	-	4	+	4	+	4	+	4	+	4	+	Absc.	+	Died	+
8	Control	-	4	+	4	+	4	+	4	+	3	+	2	+	Absc.	+

\*Rabbits inoculated intradermally with 0.6 c.c. of eighteen-hour hormone broth culture.

\*\*Divided into two doses six hours apart daily for five days; first dose given twenty-four hours after inoculation.

\*\*\*1 = severe lesion; 3 = less severe, etc., absc. = abscess.

since the doses were the same. The fact that the free sulfanilamide remains about the same in both animals, despite decreased elimination in the nephritic rabbit and the greatly increased combined sulfanilamide in the latter, can be explained if it is assumed that conjugation takes place while circulating through the fixed tissues. The latter site of conjugation would tend to depress the maximum values found for free sulfanilamide despite decreased elimination because of continual conjugation, and would cause a corresponding increase as well as a somewhat delayed appearance of combined sulfanilamide, a condition actually found to exist.

#### HISTOLOGIC EVIDENCES OF TOXICITY OF SULFANILAMIDE

All of the animals given ten daily doses of 0.004 to 0.160 gm per kg subcutaneously and 0.2 to 2.0 gm per kg orally were sacrificed after the tenth dose of the compound, and the kidneys, liver, spleen, adrenal glands, heart, and lungs examined histologically for evidences of injury, as this has been found advisable in chemotherapeutic research instead of depending solely on the duration of the lives of experimental animals for estimating the toxicity of chemical agents.<sup>6</sup> The same organs were submitted to histologic examination from the two rabbits succumbing to the oral administration of the compound.

The results were essentially negative and confirmatory of the remarkably low toxicity of sulfanilamide. None showed any histologic evidences of injury in the heart, lungs, adrenal glands, or spleen. The kidneys of a rabbit, given ten daily doses of 0.160 gm per kg by subcutaneous injection, showed slight degrees of cloudy swelling of the epithelium of the straight tubules as likewise those given 1.0 and 2.0 gm orally per kilogram of weight and succumbing on the eighth and fourth days, respectively. The liver of a rabbit receiving ten daily doses of 0.160 gm subcutaneously per kilogram and that of the rabbit given three daily doses of 2.0 gm by oral administration likewise showed marked cloudy swelling of the lobular epithelium, particularly well marked about the central veins, we are not at all sure that these changes were produced by sulfanilamide, since they have been found in control rabbits and those subjected to streptococcus infection.

#### EFFECT OF SULFANILAMIDE IN THE TREATMENT OF STREPTOCOCCUS INFECTIONS OF RABBITS

In one experiment ten rabbits were given an injection of 0.6 cc of eighteen hour hormone broth culture of virulent hemolytic streptococcus in the abdominal skin. Severe local lesions yielding positive cultures resulted in twenty-four hours, at which time treatment with sulfanilamide was begun on eight of the animals, the doses per kilogram and routes of administration being shown in Table I. Two were kept as untreated controls.

It will be observed that all receiving 0.004 to 0.08 gm subcutaneously and 0.2 to 1.0 gm orally per kilogram of weight daily for five days in succession, showed well marked clinical effects as compared with the untreated controls, but positive cultures of the lesions were secured throughout a period of ten days,

indicating incomplete disinfection of these local lesions. Two of the treated and one of the control animals succumbed, and the balance developed abscesses, five to seven days after inoculation.

In a second experiment eighteen rabbits were inoculated intradermally with the same amount of culture and at the same time given 0.5 c.c. intravenously. Twelve were subjected to treatment, the doses and routes of administration being given in Table II, while six were kept as untreated controls. The primary purpose of this experiment was to determine the effects of sulfanilamide on the prevention of suppurative arthritis.

TABLE II

EFFECT OF SULFANILAMIDE UPON THE LOCAL SKIN LESIONS AND IN THE PREVENTION OF ARTHRITIS OF RABBITS\*

NO.	DAILY DOSE PER KG.** (G.M.)	ROUTE OF ADMIN.	24 HR. AFTER IN-OCULATION; LOCAL LESIONS***	LOCAL SKIN LESIONS	EFFECT OF TREATMENT IN PREVENTION OF SUPPURATIVE ARTHRITIS
1	0.004	Subcut.	2	Regressed; died 7 days	Developed (6 days)
2	0.004	Subcut.	2	Regressed; died 7 days	Developed (6 days)
3	0.008	Subcut.	3	Healed 8 days; survived	Absent
4	0.008	Subcut.	2	Healed 9 days; survived	Developed (7 days)
5	0.08	Subcut.	3	Healed 6 days; survived	Absent
6	0.08	Subcut.	4	Healed 6 days; died 10 days	Developed (6 days)
7	0.2	Orally	3	Healed 7 days; survived	Absent
8	0.2	Orally	2	Healed 6 days; survived	Developed (8 days)
9	0.5	Orally	2	Healed 6 days; survived	Absent
10	0.5	Orally	2	Regressed; died 10 days	Developed (8 days)
11	1.0	Orally	2	Regressed; died 6 days	Absent
12	1.0	Orally	3	Regressed; died 13 days	Absent
13	Control	-	3	Progressed; died 6 days	Developed (6 days)
14	Control	-	3	Progressed; died 15 days	Developed (7 days)
15	Control	-	2	Regressed; survived	Absent
16	Control	-	2	Progressed; died 6 days	Developed (6 days)
17	Control	-	1	Progressed; died 7 days	Developed (6 days)
18	Control	-	3	Regressed; survived	Developed (7 days)

\*Rabbits inoculated with 0.6 c.c. of eighteen-hour hormone broth culture intradermally and 0.5 c.c. intravenously.

\*\*Divided into two doses six hours apart daily for five days; first dose given twenty-four hours after inoculation.

\*\*\*4 = severe lesion; 3 = less severe, etc.

Of the six untreated controls the local lesions progressed in five, and all but one, or 83 per cent, developed arthritis; four of the animals succumbed.

Of the twelve animals in which treatment was started twenty-four hours after inoculation, consisting of five daily doses of 0.004 to 0.08 gm. subcutaneously and 0.2 to 1.0 gm. orally per kilogram of weight, the local lesions regressed or healed in all; six, or 50 per cent, developed arthritis, and six, or 50 per cent, succumbed.

In a third experiment a series of rabbits were inoculated intradermally with 0.6 c.c. of eighteen-hour hormone broth culture and given 0.5 c.c. intravenously. One week later fifteen had developed arthritis, involving one to three joints. At this time treatment was started on twelve, while three were kept as controls. Six were given five daily subcutaneous injections of 0.004 to 0.08 gm. per kg. of weight, each amount being divided into two doses, six hours apart.

The purpose of the experiment was to determine the therapeutic effects of sulfanilamide upon the arthritis, and the results are shown in Table III. Joint and blood cultures were made daily.

TABLE III

EFFECT OF SULFANILAMIDE UPON THE TREATMENT OF SUPPURATIVE ARTHRITIS OF RABBITS\*

NO	NUMBER JOINTS DEVELOPING IN FLECTION 1 WEEK AFTER INOCULATION	DAILY DOSE PER KG ** (GM)	ROUTE OF ADMIN	JOINT LESIONS***	BLOOD CUL- TURES AT TIME OF DEATH
1	2	0.004	Subcut	Progressed, died 20 days	+
2	3	0.001	Subcut	Progressed, died 19 days	+
3	3	0.008	Subcut	Progressed, died 10 days	+
4	3	0.008	Subcut	Progressed, died 14 days	-
5	2	0.08	Subcut	Progressed, died 10 days	not made
6	1	0.08	Subcut	Progressed, died 13 days	+
7	3	0.2	Orally	Progressed, died 14 days	+
8	3	0.2	Orally	Regression, lived indefinitely	0
9	3	0.5	Orally	Regression, lived indefinitely	0
10	3	0.5	Orally	Progressed, died 20 days	+
11	1	1.0	Orally	Progressed, died 18 days	-
12	1	1.0	Orally	Regression, lived indefinitely	0
13	3	Control	0	Progressed, died 10 days	+
14	2	Control	0	Progressed, died 17 days	+
15	3	Control	0	Progressed, died 11 days	+

\*Rabbits inoculated with 0.6 cc of eighteen hour hormone broth culture intradermally and 0.5 cc intravenously.

\*\*Divided into two doses six hours apart daily for five days; first dose given seven days after inoculation.

\*\*\*Joint cultures made every two to three days after development of arthritis; all positive.

The arthritis of all three untreated controls progressed, and all three succumbed in ten to thirteen days after inoculation with positive joint and blood cultures.

Of the twelve treated animals, the arthritis continued to progress in nine, or 75 per cent, nine, or 75 per cent, succumbed with positive blood and joint cultures. Arthritis regressed in three (25 per cent), and the animals lived indefinitely, although positive joint cultures persisted throughout a period of twenty-one days following inoculation.

#### EFFECT OF SULFANILAMIDE UPON PHAGOCYTOSIS AND CONCENTRATION OF THE COMPOUND IN THE BLOOD AND LOCAL LESIONS OF RABBITS

In one experiment six rabbits were inoculated intradermally with 0.6 cc of eighteen hour hormone broth culture. Twenty-four hours later severe local lesions had developed and smears showed that from 12 to 25 per cent of the polymorphonuclear leucocytes had become phagocytic.

Three of the animals were now given 0.004 to 0.08 gm sulfanilamide by subcutaneous injection, and two 0.1 and 0.5 gm by oral administration per kilo gram of weight, each dose being divided into two portions and given six hours apart (Table IV). One animal was kept as an untreated control.

Twenty-four hours later the local lesions of four of the five treated animals showed clinical regression, and five showed an appreciable degree of increased phagocytosis by the polymorphonuclear leucocytes of the inflammatory exudates.



TABLE IV  
EFFECT OF SULFANILAMIDE UPON PHAGOCYTOSIS AND CONCENTRATION OF THE COMPOUND IN THE BLOOD AND LOCAL LESIONS\*

NO.	LESION 24 HOURS AFTER INOCULATION			TREATMENT***		LESION 24 HOURS LATER			SULFANILAMIDE; MG. PER 100 C.C.	
	CLINICAL	CULTURE	PHAGO- CYTOSIS***	DOSE PER KG. (MG.)	ROUTE ADMIN.	CLINICAL	CULTURE	PHAGO- CYTOSIS***	BLOOD	LESION
1	4**	+	12	0.004	Subcut.	2**	+	16	0.5	Trace*****
2	4	+	12	0.008	Subcut.	2	+	25	0.2	Trace
3	4	+	25	0.08	Subcut.	4	+	26	0.2	Trace
4	4	+	12	0.1	Orally	3	+	12	0.3	Negative
5	4	+	12	0.5	Orally	3	+	18	0.8	1.3
6	4	+	16	Control	-	4	+	12	Negative	Negative

\*Rabbits inoculated with 0.6 c.c. of eighteen-hour hormone broth culture intradermally.

\*\*1 = severe lesion; 3 = less severe, etc.

\*\*\*Percentage of polymorphonuclear leucocytes in lesion smears found phagocytic.

\*\*\*\*Divided into two doses six hours apart.

\*\*\*\*\*Less than 0.1 mg. per 100 c.c.

Free sulfanilamide was found in the blood, varying from 0.2 to 0.8 mg per 100 cc

The lesions were now excised, weighed, ground with washed sand, and extracted in an incubator at 37° C for twenty-four hours with 95 per cent alcohol. After paper filtration, an aliquot portion of each filtrate was tested for free sulfanilamide. The lesions of three of the treated animals showed traces of free sulfanilamide (less than 0.1 mg per 100 gm); one was negative, and one showed 1.3 mg per 100 gm (rabbit No. 5 received 0.5 gm orally per kilogram of weight).

Smears of the local lesions were made twenty-four hours after inoculation in the case of the 8 rabbits listed in Table V. From 4 to 28 per cent of the polymorphonuclear leucocytes were found phagocytic.

TABLE V

EFFECT OF SULFANILAMIDE UPON PHAGOCYTOSIS OF STREPTOCOCCI IN LOCAL SKIN LESIONS OF RABBITS\*

NO	DAILY DOSE IFT AC** (gm)	ROUTE OF ADMIN	PER CENT PHAGOCYTIC CELLS IN FLOUDES, DAYS						
			4 HRS AFTER INOCULATION EFFORT TREATMENT	2	3	4	5	7	10
1	0.004	Subcut	10**	48	6	6	16	8	10
2	0.008	Subcut	8	10	24	36	10	22	Died
3	0.08	Subcut	12	14	36	38	40	14	14
4	0.2	Orally	16	28	24	8	24	10	14
5	0.5	Orally	4	16	20	32	30	16	12
6	1.0	Orally	20	16	22	Died			
7	Control	-	12	8	14	20	12	8	10
8	Control	-	28	28	26	40	34	8	10

\*Rabbits inoculated intradermally with 0.6 cc of eighteen-hour hormone broth culture.

\*\*Divided into two doses six hours apart daily for five days.

\*\*\*Fifty cells counted in smears of lesion; percentage phagocytic.

Three were given subcutaneous injections of 0.004 to 0.08 gm of sulfanilamide, and three 0.2 to 1.0 gm by oral administration daily for five days in succession, each amount being divided into two doses, six hours apart. Two were kept as untreated controls.

Phagocytic counts were made daily up to ten days.

In one of the untreated controls, from 8 to 20 per cent of the polymorphonuclear leucocytes were phagocytic throughout, while in the second, from 8 to 40 per cent were phagocytic.

Among five of the six treated animals, the percentage of phagocytes was materially increased during the first one to three days of treatment, but not in the case of No. 5, which was given 1.0 gm per kg orally and succumbed three days after inoculation.

Phagocytic studies were also made in the case of all of the eighteen rabbits listed in Table II. As previously stated, these had been inoculated intradermally with 0.6 cc of culture, and intravenously with 0.5 cc.

Six were subjected to treatment with sulfanilamide in dose of 0.004 to 0.08 gm subcutaneously, and six with 0.2 to 1.0 gm per kg by oral administration daily for five days beginning twenty-four hours after inoculation, each amount being divided into two doses, six hours apart. Six animals were kept as untreated controls.

TABLE VI  
EFFECT OF SULFANILAMIDE UPON PHAGOCYTOSIS AND CULTURES OF SKIN LESIONS, BLOOD, AND JOINTS OF INFECTED RABBITS\*

NO.	DAILY DOSE PER KG.** (GM.)	ROUTE OF ADMIN.	PHAGOCYTOSIS***			LESION CULTURES					BLOOD CULTURES					JOINT CUL- TURES****
			BEFORE	DAYS		BEFORE	DAYS				BEFORE	DAYS				
				2	5		2	5	7	10		2	5	7	10	
1	0.004	Subcut.	18	6	0	+	+	-	D	-	+	+	-	D	-	+
2	0.004	Subcut.	30	24	4	+	+	-	D	-	+	-	+	D	-	0
3	0.008	Subcut.	10	18	0	+	+	-	-	-	+	-	-	-	-	0
4	0.008	Subcut.	32	28	3	+	+	+	-	-	+	-	-	-	-	0
5	0.08	Subcut.	22	8	0	+	+	+	-	-	+	-	-	-	-	0
6	0.08	Subcut.	16	4	0	+	+	+	-	-	+	-	-	-	-	0
7	0.2	Orally	10	18	2	+	+	-	-	-	+	-	-	-	-	0
8	0.2	Orally	10	6	0	+	+	-	-	-	+	-	-	-	-	0
9	0.5	Orally	30	18	6	+	+	+	-	-	+	+	-	-	-	0
10	0.5	Orally	10	10	0	+	+	+	D	-	+	+	-	D	-	0
11	1.0	Orally	34	18	0	+	+	+	-	-	+	+	-	-	-	0
12	1.0	Orally	20	10	0	+	+	+	-	-	+	+	-	-	-	0
13	Control	-	30	11	-	+	+	+	-	-	+	+	+	+	+	+
14	Control	-	35	8	0	+	+	+	D	-	+	+	+	D	+	+
15	Control	-	16	16	8	+	+	+	-	-	+	+	+	+	+	0
16	Control	-	16	12	6	+	+	+	+	-	+	+	+	+	+	+
17	Control	-	14	12	0	+	+	+	D	-	+	+	+	D	+	+
18	Control	-	12	12	4	+	+	+	+	+	+	+	+	+	+	+

\*Rabbits inoculated with 0.6 c.c. of eighteen-hour hormone broth culture intradermally and 0.5 c.c. intravenously.

\*\*Divided into two doses six hours apart daily for five days; first dose twenty-four hours after inoculation.

\*\*\*Percentage of polymorphonuclear leucocytes in lesion smears found phagocytic; 0 = not made.

\*\*\*\*Cultures made at same intervals as cultures of lesions and blood; + = positive culture; 0 = no arthritis and culture not made.

Twenty-four hours after inoculation smears of the exudates of the local lesions showed that from 10 to 35 per cent of the polymorphonuclear leucocytes had become phagocytic (Table VI).

Of the six untreated controls, the percentage of phagocytes dropped in the following five days. The local lesion and blood of the one animal (No. 15) became spontaneously sterile on the seventh day, and this animal escaped arthritis and lived indefinitely, but all of the remaining five developed arthritis and showed positive skin lesion, blood, and joint cultures throughout the period of observation, three succumbing about seven days after inoculation.

Of the six receiving five daily subcutaneous injections of sulfanilamide in dose of 0.004 to 0.08 gm., and six from 0.2 to 1.0 gm. by oral administration per kilogram of weight, only two showed an increase of phagocytosis.

Five, or 42 per cent, of the twelve treated animals succumbed as compared to three, or 50 per cent, of the six untreated controls. Ten, or 83 per cent, of the treated animals gave sterile lesion cultures as compared with 17 per cent of the untreated controls; and ten, or 83 per cent, gave sterile blood cultures, although five developed arthritis and four succumbed.

#### DISCUSSION

These results have confirmed the observations of others in regard to the remarkably low toxicity of sulfanilamide for the normal animal as determined not only by the duration of life and the absence of demonstrable toxic effects, but likewise by the absence of pronounced histologic changes in the various organs following the administration of large amounts per kilogram of weight. They have also shown the rapid absorption of the compound from the subcutaneous tissues and the gastrointestinal tract and its rapid elimination requiring frequent administration for the maintenance of effective amounts in the blood. They have also shown that defective elimination by the kidneys of both free and conjugated sulfanilamide as the result of nephritis materially increases toxicity and indicates smaller dosage and extra caution in the treatment of human beings under these conditions. In other words, sulfanilamide in ordinary dosage does not appear to increase the degree of nephritis, but its toxicity is increased by reason of defective elimination.

Evidence is presented indicating that conjugation of sulfanilamide probably occurs in the blood and the fixed tissues, but the question of its therapeutic properties is uncertain at the present time. If therapeutic effectiveness depends primarily upon free sulfanilamide in the blood, almost toxic and lethal amounts must be given the normal rabbit to attain a concentration of 10 to 12 mg. per 100 c.c., believed to be required in the treatment of human beings.

Our results have shown that sulfanilamide exerts a well-marked clinical effect upon the local skin lesions in rabbits produced by the intradermal injection of virulent beta hemolytic streptococci, but in the doses employed it has only occasionally and irregularly effected complete sterilization of the lesions. It has also demonstrated some effect in the sterilization of the blood and in the prevention of secondary infection of the joints. Probably the duration of treatment employed in these experiments has been insufficient, although the dosage per kilogram of body weight has been in most instances materially greater than

employed in the treatment of human beings. But at all events, it appears that there is need for a compound possessing a greater capacity for disinfection or sterilization of the primary and secondary infections of the fixed tissues than demonstrated by sulfanilamide in these experimental infections of rabbits.

While the mechanism of curative activity of sulfanilamide has not yet been completely elucidated, the results observed in this study tend to confirm the opinion that at least part of its therapeutic effectiveness is due to a promotion of phagocytosis of streptococci in vivo probably by reason of exerting sufficient injury or bacteriostasis of the organisms and destruction of toxins to reduce their antiphagocytic properties. In a recent study of sulfanilamide in the treatment of experimental streptococcus and pneumococcus meningitis of rabbits and monkeys by Kolmer, Rule, and Werner,<sup>7</sup> there was likewise distinct evidence indicating that the compound promoted phagocytosis in the purulent cerebrospinal fluids, and while the amounts of the latter were insufficient for quantitative tests for the presence of the compound, the results indicated that the promotion of phagocytosis was apparently an important phase of therapeutic activity.

#### SUMMARY AND CONCLUSIONS

1. Sulfanilamide by subcutaneous injection is remarkably low in toxicity for normal rabbits, since five daily doses, as high as 0.160 gm. per kg., were without toxic effects.

2. Ten doses at the rate of two per day by oral administration gave a maximum tolerated dose of about 0.3 gm. per kg. of weight.

3. The compound is also remarkably low in the production of pathologic tissue changes in the normal rabbit. Only the repeated administration of such large amounts as 0.160 gm. subcutaneously and 1.0 to 2.0 gm. orally per kilogram of weight, produced suggestive cloudy swelling of the tubular epithelium of the kidneys and the lobular epithelium of the liver.

4. Nephritis of rabbits materially reduces tolerance for sulfanilamide, and the compound should be given in reduced dosage and with extra caution to human beings with defective kidney elimination.

5. The compound is rapidly absorbed from the subcutaneous tissues and gastrointestinal tract of rabbits. In one-half hour after oral administration considerable free sulfanilamide can be found in the blood, and maximum amounts of both free and conjugated sulfanilamide are found after two to three hours. Little or no conjugated sulfanilamide is present in the blood as early as one-half hour after oral administration.

6. Elimination is also quite rapid in the normal rabbit since only a negligible amount of free sulfanilamide is found in the blood after about thirty-two hours. In nephritic rabbits, however, elimination is materially delayed and conjugated sulfanilamide accumulates to a greater extent than free sulfanilamide with an increase of toxicity.

7. The maintenance of 10 to 12 mg. of free sulfanilamide per 100 c.c. of blood, believed to be required for the maximum of therapeutic effectiveness, requires in the rabbit the administration of almost maximum tolerated or toxic amounts of the compound.

8 Conjugation of sulfanilamide appears to occur in the blood and fixed tissues, but its therapeutic effectiveness is uncertain and unknown at the present time

9 The intradermal inoculation of rabbits with virulent beta hemolytic streptococcus produces a severe local lesion without septicemia available for clinical observation and bacteriologic examination for evaluating the therapeutic effectiveness of chemical agents. The simultaneous intravenous injections of culture results in the production of septicemia and a large percentage of animals develop suppurative arthritis. These lesions are believed to be more useful than streptococcus infections of mice for chemotherapeutic investigations

10 Sulfanilamide in the doses employed usually produced prompt and marked clinical regression of the local streptococcus skin lesions, but only irregularly and occasionally complete bactericidal effects. Small amounts of free sulfanilamide were found in these lesions but not in the amounts regarded as required for sterilization of the blood

11 About 83 per cent of untreated control rabbits developed arthritis, and 50 per cent developed this secondary infection under sulfanilamide treatment

12 In all untreated control rabbits arthritis progressed and all died with positive blood and joint cultures. In a series treated with sulfanilamide the joint lesions continued to progress in 75 per cent, and 75 per cent succumbed with positive blood and joint cultures

13 Sulfanilamide usually increased the phagocytosis of streptococci in the local skin lesions, and it appears that at least part of the therapeutic effectiveness of the compound consists in producing sufficient bacteriostasis and destruction of toxins to promote phagocytosis

14 While sulfanilamide represents a marked advance in the chemotherapy of hemolytic streptococcus infections, especially of septicemia, there is urgent need for the production of compounds more effective in the disinfection or sterilization of the primary and secondary fixed tissue infections

#### REFERENCES

- 1 Kolmer, J. A., and Rule, A. M. Experimental Streptococcus Infections in Rabbits for Therapeutic Investigations, *J. LAB & CLIN. MED.* 22: 1097, 1937
- 2 Kolmer, J. A., Brown, H., Ruiziss, G. W., Rule, A. M., and Clemence, L. W. The Chemotherapy of Experimental Streptococcus Infections of Rabbits With Special Reference to Pyridine Compounds and Prontosil Soluble, *J. Pharmacol. & Exper. Therap.* 61: 253, 1937
- 3 Ruiziss, G. W., Severac, M., and Moetsch, J. C. Chemotherapeutic Studies of Sulfamidyl (Para Amino Benzene Sulfonamide) in Experimental Beta Hemolytic Streptococcal Infection, *J. Chemotherapy* 14: 1, 1937
- 4 Marshall, E. K., Emerson, K., and Cutting, W. C. Para Aminobenzenesulfonamide, Absorption and Excretion. Method of Determination in Urine and Blood, *J. A. M. A.* 108: 953, 1937
- 5 Marshall, E. K. Determination of Sulfanilamide in Blood and Urine, *J. Biol. Chem.* 122: 263, 1937
- 6 Kolmer, J. A. Principles and Practice of Chemotherapy With Special Reference to the Treatment of Syphilis, Philadelphia 1926, W. B. Saunders Co., p. 601
- 7 Kolmer, J. A., Rule, A. M., and Werner, M. F. Sulfanilamide in the Treatment of Experimental Streptococcus and Pneumococcus Meningitis, *Arch. Otolaryng.* 27: 519, 1938

# LABORATORY METHODS

## A RAPID AND SIMPLIFIED METHOD OF EXTRACTING URINARY ESTROGEN\*

S. L. LEIBOFF, M.A., AND ABRAHAM B. TAMIS, M.D., NEW YORK, N. Y.

THE biochemical estimation of estrogenic substance is admittedly an important step in the study of functional disorders of the female genital tract. The more general adoption of such hormone studies will depend primarily upon the rapidity and simplicity of the procedure. The improved apparatus described herein meets these requirements. The extraction of urinary estrogen is completed within three hours, and has given us results which compare favorably in every way to other methods.

Prior to 1932, two procedures were commonly used; namely, the chloroform method of Frank and Goldberger,<sup>1</sup> and the ethyl acetate method of Clarke and Kurzrok.<sup>2</sup> The latter method has been used in our laboratory for the past five years because it proved to be more easily assembled and required less attention. The duration in which the droplets of ethyl acetate were in contact with the urine was so short that it was necessary to continue the extraction for twenty-four to forty-eight hours to secure complete extraction of the estrogenic substance. In 1935, Smith and Smith<sup>3</sup> advocated a benzene method which they believed gave a more efficient estrogen assay than chloroform, olive oil, or ethyl acetate. The fire hazard with benzene requires the extraction to be conducted under extreme precautions, a serious handicap to its general use. These authors stressed the importance of subjecting the urine to hydrolysis in order to release any combined estrogen. It is very likely that this preliminary treatment of the urine accounts for the greater efficiency of the Smith and Smith procedure.

Estrogenic substance is excreted in a combined form to a considerable extent in pregnancy urine, and to a much less extent in the urine of nonpregnant mature females. In this combined form it is fat-insoluble and biologically inert.<sup>4</sup> By subjecting the urine to bacterial hydrolysis (permitting the urine to incubate for forty-eight hours or remain at room temperature for three days), or by boiling the urine with acid (acid hydrolysis), the combined estrogen is "freed" and made extractable by the fat solvent.

Our experience with acid hydrolysis of the urine preliminary to using the Clarke and Kurzrok set-up was very unsatisfactory because the ethyl acetate was broken down into acetic acid by the treated urine. This occurred in spite of cooling and neutralizing the urine before setting up the extraction.

\*From the Department of Laboratories, Lebanon Hospital, New York.  
Received for publication, March 2, 1938.

In seeking for another method, we combined the advantages of all the aforementioned procedures and developed the new apparatus shown in Fig. 1.

#### METHOD

The apparatus is assembled as in Fig. 1 with the exception of the condenser *D*. The Erlenmeyer flask is of 100 c.c. capacity and is connected to the arm *F* by a cork stopper.

Sufficient chloroform is placed into the extractor *B* to reach a level about 2 inches higher than the glass disk *C*.

Five hundred cubic centimeters of a measured twenty-four-hour collection of urine are acidified with 50 c.c. of concentrated hydrochloric acid, and the

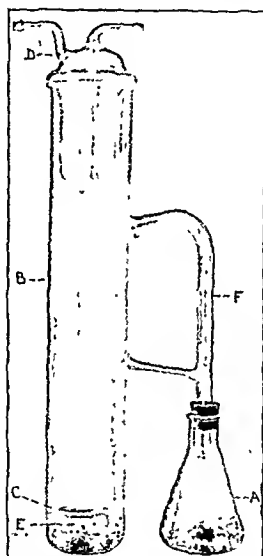


Fig. 1.

urine evaporated to one-third its volume by boiling. It is then cooled and poured into the extractor *B* through a long-stemmed funnel. The urine will form a layer above the chloroform.

Additional chloroform is slowly poured into the extractor *B* until the chloroform is seen to pass through tube *E* into flask *A*. Enough chloroform is added to allow about 25 c.c. of chloroform to collect in the flask.

The condenser *D* is set in place and the cold water turned on. Heat is then applied to the extractor and the flask.

When the chloroform in the extractor is brought to a boil, at first it rises partly into the layer of urine and slowly drops back again. When the tempera-



ture of the urine is elevated to that of the boiling chloroform, the chloroform will bubble vigorously through the layer of urine and vaporize into the column of air above where it is reliquefied by the condenser. At this stage an emulsion will form between the layer of chloroform and the urine. The flame must now be lowered to prevent the emulsion from entering the tube *E*. Once this point is reached the apparatus need not be watched any longer until the extraction is completed.

While the above is taking place, the chloroform in the flask is also being vigorously heated. The chloroform vapors from the flask pass through the arm *F* into the extractor and are condensed. The increased volume of chloroform in the extractor causes the chloroform at the bottom of the extractor to rise in tube *E* and spill over into the flask. This performance is a continuous one.

Within two hours all the chloroform-soluble substances of the urine have been completely extracted and transferred to the flask.

The flask is then disconnected. Five cubic centimeters of propylene glycol (olive oil or cotton seed oil may be used instead) are added to the chloroform extract, and the chloroform driven off by heating over a water bath until no odor of chloroform is detected. The remaining extract is used for assay purposes, 1 c.c. of the extract representing 100 c.c. of urine.

#### SUMMARY

A new apparatus with a high efficiency of extraction of urinary estrogen is described. The period of extraction is reduced to less than three hours. The method advocated can be readily adopted by any laboratory because of its simplicity and its safety.

The authors wish to express their appreciation to Dr. Joseph C. Ehrlich, Director of Laboratories, for his helpful assistance in the elaboration of the apparatus described herein. We are also indebted to the Empire Laboratory Supply Co., New York City, for their generous cooperation in the development of the apparatus.

#### REFERENCES

1. Frank, R. T.: The Role of the Female Sex Hormone, *J. A. M. A.* 97: 1852, 1931.
2. Kurzrok, R., and Ratner, S.: The Relation of Amenorrhea Accompanied by Genital Hypoplasia to the Follicular Hormone in the Urine, *Am. J. Obst. & Gynec.* 23: 689, 1932.
3. Smith, G. V. S., and Smith, O. W.: Quantitative Determination of Urinary Estrogenic Substance, *Am. J. Physiol.* 112: 340, 1935.
4. Cohen, S. L., Marrian, G. F., and Watson, M.: Excretion of Estrin During Pregnancy, *Lancet* 1: 674, 1935.

## THE STABILITY OF KLINE ANTIGEN EMULSIONS\*

EDWARD L. BRFAZEALF, B.S.A., AND ROBERT A. GRFENE, PH.D., TUCSON, ARIZ

THE Kline precipitation test for syphilis is widely employed because of its simple technique, accuracy, and relatively low cost. One tenth milliliter of antigen will yield sufficient antigen emulsion for several hundred diagnostic tests. Since the antigen emulsion is considered as satisfactory for use for only forty eight hours after preparation, there are many laboratories which will use only a small portion of the antigen emulsion within this time.

A lot of the diagnostic antigen emulsion was prepared on August 19, 1937, and was placed in an icebox by error. The emulsion was discovered several days later and was tested against known positive and negative sera, the results agreed perfectly with those which were obtained with freshly prepared antigen emulsions. This lot of emulsion was stored in an icebox and was checked at frequent intervals, reliable results were obtained for four months, after that, there was a progressive increase in sensitivity, and at the end of five months, it gave strongly positive reactions with negative sera. In the meantime, other lots of emulsion have been treated in the same way, and they have given reliable results for relatively long periods.

It would appear that Kline diagnostic antigen emulsions, when stored in an icebox, are relatively stable and may be used for at least several weeks. Naturally, the antigen emulsion should be tested against known positive and negative sera prior to use.

## AN INEXPENSIVE PORTABLE ELECTRICAL THERMOMETER FOR THE HYPERPYREXIA CLINIC†

FRANCIS W. BISHOP, ROCHESTER, N. Y.

IN ROUTINE hyperpyrexia it is desirable to employ some type of continuously indicating thermometer. This is especially true in the case of prolonged fever bouts (ten to twenty four hours). The use of such an instrument spares the patient a great deal of trauma and, on the whole, greatly facilitates the accurate administration of the fever. Of the electrical instruments for this purpose, either the thermocouple or the resistance thermometer may fulfill the requirements. The principal disadvantages of these devices have been the bulk, complexity, and cost. For the past six years, we have used both

\*From the Arizona State Laboratory Tucson

†From the Department of Medicine Division of Radiology of the University of Rochester School of Medicine and Dentistry and Strong Memorial Hospital Rochester

Received for publication March 3 1938

thermocouple and resistance thermometers for the rectal thermometry, but the instrument of our choice has been the resistance thermometer.

We have attempted to construct a suitable instrument at the lowest possible cost, and it is our purpose to describe a resistance thermometer unit which may be duplicated in most laboratories for \$75.00. One of the principal difficulties was to find a galvanometer of the necessary accuracy and sensitivity

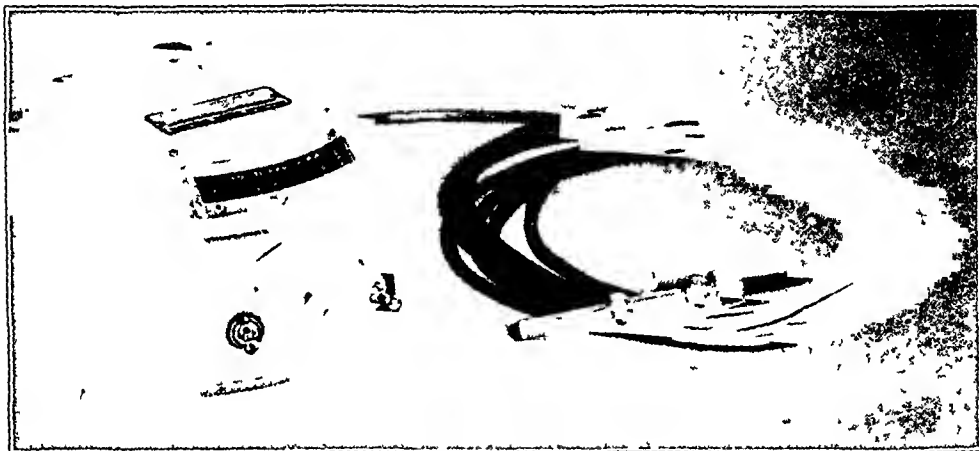


Fig 1—The thermometer is shown completely assembled. Adjustment shafts for  $R_1$  and  $R_2$  are in back of case. Note. External finding posts are standard with this galvanometer unless otherwise specified

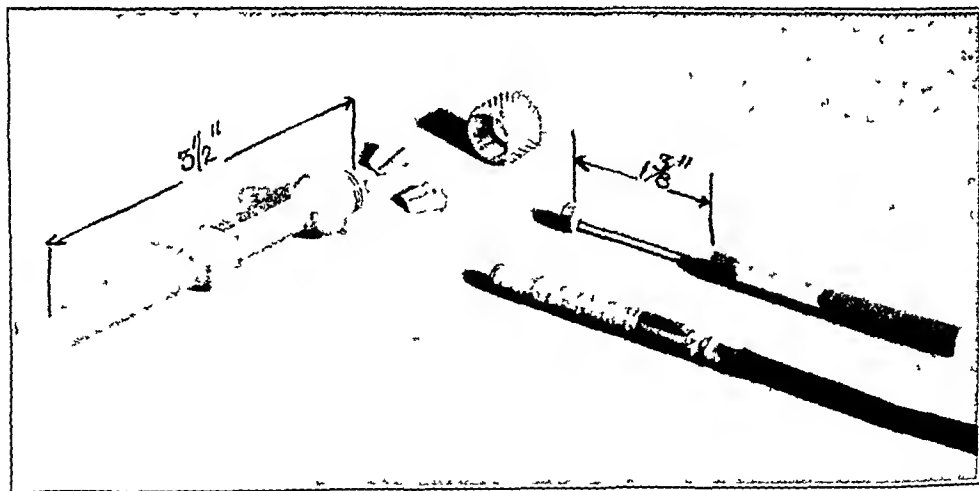


Fig. 2—This figure shows the steps of assembly of temperature—responsive element and split cone-locking device to prevent cable from being pulled out of its housing. Winding is shellacked and housing filled with wax to reduce thermal lag.

which was not too expensive. This has been very well met in the G-M No. 2562-B pointer-type D'Arsonval galvanometer, costing \$20.00 (Fig. 1). This instrument has a resistance of 300 ohms, a sensitivity of 0.25 microampere per mm. deflection, and a 60 mm. scale division, which is calibrated in degrees Centigrade from 36.0° to 42.0°. Each millimeter scale division thus equals

01° C The galvanometer is of the taut suspension type, and is more rugged and less susceptible to damage than the ordinary jewel bearing meter. They may be obtained in various sensitivities to suit the resistance thermometer used. The thermometer bulb itself consists of a coil of nickel having a resistance of about 600 ohms (569.21 at 36.0° C to 609.05 at 42.0° C in one case). The wire is wound on an insulating bobbin and is placed in a thin brass housing of the proper size and shape to be retained in the rectum (Fig 2). This item can probably be purchased or constructed for \$20.00 or less. In the bridge circuit in Fig 3, resistances  $R_1$  and  $R_2$  are Leeds and Northrup manganin wire unmounted resistors, costing \$1.50 each. Resistor  $R_3$  is specially wound to

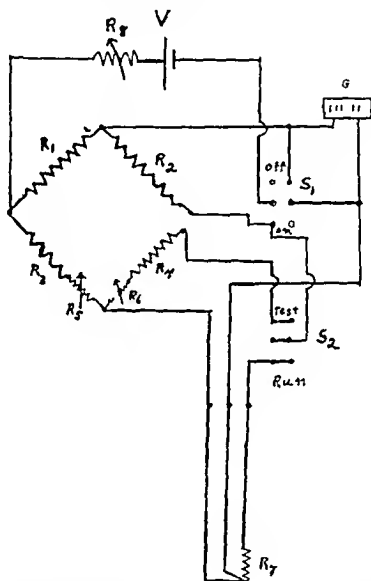


Fig 3—Complete wiring diagram of G No 2562 G M 300 ohm pointer type 4584 unmounted resistor,  $R_2$  500 ohm L Leeds and Northrup No 4584S unmount  $R_4$  Leeds and Northrup No 4584S unmo 42.0° C  $R_5$  5 ohm wire wound radio co 100 ft No 40 nickel wire wound on suitable form—resistance between 500 and 600 ohms  $R_6$  500 to 1000 ohm wire wound radio control and  $S_1$  and  $S_2$  double pole double throw radio tog

1 No 950 flashlight cell  
Leeds and Northrup No  
unmounted resistor  $R_3$   
an  $R_1$  at 0 or 36.0° C  
than  $R_1$  at full scale or  
radio control  $R_1$  about

have a resistance of  $2\frac{1}{2}$  ohms less than the resistance of the thermometer at 36.0° C, while resistor  $R_4$  is adjusted to  $2\frac{1}{2}$  ohms less than the resistance of the thermometer at 42.0° C

Resistors  $R_1$  and  $R_6$  are ordinary 5 ohm wire wound variable radio rheostats. The shafts of these rheostats are slotted for adjustment with a screw driver, and once set are not disturbed. Variable rheostat  $R_3$  is wire wound, has a resistance of 500 to 1,000 ohms, and is used to compensate for any

change in the battery voltage. Battery "V" consists of one No. 950 flashlight cell. The current drain is 2.5 Ma., which should provide some months of ordinary use. Flashlight cells are used in order to eliminate any external batteries or connections, and so to make the unit entirely portable and self-contained. There are two small double-pole, double-throw toggle switches. One of these is used to turn the unit off or on and when this switch is in the "off" position, the galvanometer is short-circuited. The second switch is used to connect either the thermometer or the test resistance  $R_4$  to the bridge.

To calibrate the resistance thermometer, the bulb ( $R_7$ ) is placed in a water bath at  $36.0^\circ \text{C}$ . The switch is placed in "run" position, and resistance  $R_5$  is adjusted to bring the galvanometer to zero or  $36.0^\circ \text{C}$ . The temperature of the thermometer is raised to  $42.0^\circ \text{C}$ . and resistance  $R_8$  is adjusted to bring the galvanometer reading to  $42.0^\circ \text{C}$ ., or full scale. The switch is then thrown to "test" position, and resistance  $R_6$  is changed until the galvanometer reads  $42.0^\circ \text{C}$ . Resistances  $R_5$  and  $R_6$  are not further changed, the galvanometer deflection being standardized at any time simply by throwing the switch to "test" position, and then, if necessary, by moving the control rheostat  $R_8$  to bring the needle to  $42.0^\circ \text{C}$ . The switch is then thrown to "run" position, and the galvanometer deflection will indicate the temperature of the thermometer bulb if its temperature is between  $36.0^\circ \text{C}$ . and  $42.0^\circ \text{C}$ .

We attempted to use good quality radio resistors throughout, but these were so affected by ambient temperature and humidity changes as to be useless. Resistors  $R_5$  and  $R_6$ , however, comprise such a small proportion of the total resistance as to be negligible in their variations. The other fixed resistors,  $R_1$ ,  $R_2$ ,  $R_3$ , and  $R_4$ , should be high quality manganin coils. The resistances of the arms of the bridge and of the thermometer coil should be fairly high in order to keep the drain on the battery low. The temperature responsive element is connected to the bridge by a small flexible three-wire cable. This connection shown in Fig. 3, eliminates the effects of changes in resistance of the leads with temperature. The unit may be calibrated in degrees Fahrenheit or Centigrade, and special scales are obtainable from the manufacturer of the galvanometer. If temperature control is desired by means of the above-described apparatus, the galvanometer may be substituted by the G-M Super-Sensitive D'Arsonval galvanometer relay, with no loss of sensitivity. This instrument includes the same galvanometer as before, but with a synchronous motor driven contacting device, which may be used with a secondary relay for the automatic control of the patient's temperature.

This unit, in common with other electrical thermometers, will not function properly where high frequency fields are employed to produce the fever.

A simple electrical thermometer has been described, the cost of which brings it within the reach of any hyperpyrexia clinic.

# THE COMPLEMENT FIXATION TEST IN CHANCROIDAL INFECTION\*

EVERETT S. SANDERSON, PH D, M D, ROBERT B. GREENBLATT M D, C M,  
AND ELIZABETH BATHUR, B A, AUGUSTA GA

ELSEWHERE<sup>1</sup> we have described the results of the skin test, utilizing a bacillary antigen, in the differentiation of chancroidal infection. From a clinical standpoint this test has proved very satisfactory. The one drawback, if such it may be called, is due to the fact that the test having to be read forty eight hours after injection of antigen, necessitates a revisit to the clinic on the part of the patient. Sometimes this is a hardship, and sometimes the patient fails to return, with the result in such cases that the test must be repeated.

Positive skin reactions in chancroidal infection are undoubtedly antigen antibody combinations, and so it was felt that some *in vitro* serologic test might be of equal value, and thereby eliminate the revisit of the patient. Owing to the methods we found to be essential in the cultivation of *H. ducreyi*, together with the peculiarities in the growth of this organism, an agglutination test was out of the question. The complement fixation test suggested itself as an alternative.

## METHODS†

The complement fixation test adopted utilized two units of antigen, one and one half units of complement, 0.1 cc of test serum, rabbit antsheep hemolytic system, and adjusted to a total volume of 2.5 cc with saline. Fixation employed the 37° C water bath throughout one hour preliminary, and one half hour final. Since the antigen was found to be the crux of the whole situation, its preparation will be described.

The strains of *H. ducreyi* which we had isolated grew very poorly on the surface, and it was this observation which led us to adopt the method of cultivation and preparation of skin test antigen previously described<sup>1</sup>. Essentially, this consisted of growing the organism in defibrinated human blood added onto the surface of infusion agar slants, reducing the oxygen tension by gently heating the side of the tube, and then tightly stoppering it. After three days' incubation at 37° C, the blood and growth were removed to sterile distilled water and centrifugated at high speed. The sediment was washed until the supernatant was free of hemoglobin—usually twice—and to the final sediment was added sterile physiologic saline to the extent of 10 cc per agar slant. The antigen was heated for forty minutes at 60° C, and 1:10,000 merthiolate

\*From the Department of Bacteriology and Public Health and the Department of Pathology School of Medicine University of Georgia Augusta

Received for publication March 8 1933

†In these studies our attention has been called to a publication by Schmidt and others, 409 1932. Nine cases of chancroid were studied. Details of methods and technique were obtained in all. Details of methods and technique were that Dmelcos vaccine was used as the antigen—a prep to the American market. We have reported comparative and our own preparations and the results were always similar.

TABLE I  
COMPLEMENT FIXATION TESTS

CASE	ANTIGEN (HUMAN BLOOD GROWTH)	ANTIGEN (RABBIT BLOOD GROWTH)	CHANCROID SKIN TEST	FREI TEST	REMARKS
1	2- 4 negative 3-11 negative 3-25 negative 5- 6 negative	4- 1 4 plus 4- 9 4 plus 4-15 4 plus 4-23 4 plus 5- 6 4 plus	positive	negative	Granuloma inguinale Donovan's bodies present Fusospirochetosis
2	2- 4 negative 3-11 negative	-----	positive	negative	Clinically chancroid Multiple lesions 1-14
3	3-11 negative	-----	positive	positive	Chancroid; ulceration for 6 months
4	3-11 negative	4-23 4 plus 5- 6 4 plus	positive	negative	Chancroid; ulcers a few weeks
5	3-11 negative 3-25 negative	-----	positive	negative	Clinically chancroid; ulcer at fourchette 2 weeks
6	3-25 negative	-----	positive	positive	Clinically chancroid; bubo present; had bubo 1 year ago also
7	3-25 negative	-----	negative	positive	W.R. became positive after 5 weeks; hard bubo, probably syphilitic
8	3-25 negative	4- 9 4 plus 4-15 4 plus 4-23 4 plus	negative	positive	Lymphogranuloma venereum Rectal stricture
9	3-25 negative	-----	doubtful	positive	Probably lymphogranuloma venereum. Ulcer on four- chette for 2 weeks
10	3-25 negative	4- 1 3 plus 4- 9 4 plus 4-15 4 plus 4-23 4 plus	positive	positive	Lymphogranuloma venereum Perianal abscess
11	3-25 negative	4- 1 4 plus 4- 9 4 plus 4-23 4 plus	negative	positive	Lymphogranuloma venereum Elephantiasis of vulva
13	3-25 negative	-----	negative	positive	Lymphogranuloma venereum
14	-----	4- 1 negative 4- 9 3 plus 4-15 2 plus 4-23 1 plus	negative	positive	Lymphogranuloma venereum (rectal)
15	-----	4- 1 2 plus 4- 9 4 plus	positive	weak	Probably chancroid
16	-----	4- 1 3 plus 4- 9 4 plus 4-23 4 plus	weak	positive	Probably lymphogranuloma ve- nereum. Multiple small ul- cers on cervix
17	-----	4- 1 4 plus 4- 9 4 plus	negative	negative	Acute G.C.
18	-----	4- 1 1 plus 4- 9 4 plus	negative	positive	Lymphogranuloma venereum. Bilateral buboes. No primaries

TABLE I—CONT'D

CASE	ANTIGEN (HUMAN BLOOD GROWTH)	ANTIGEN (RABBIT BLOOD GROWTH)	CHANCROID SKIN TEST	FEEI TEST	REMARKS
19	-----	4 1 negative 4 9 4 plus 4 23 3 plus	negative	positive	Clinically indefinite
20	-----	4 1 4 plus 4 9 4 plus	positive	positive	Wassermann 4 plus Clinically mixture of syphilis, lympho granuloma venereum, and chancreoid
21	-----	4 1 negative 4 9 negative	positive	weak	Clinically chancreoid
22	-----	4 1 negative	positive	negative	Clinically chancreoid Multiple penile sores
23	-----	4 1 3 plus	positive	negative	Clinically chancreoid, 7 months' duration
24	-----	4 15 4 plus 4 23 4 plus	positive	negative	Clinically granuloma inguinale Donovan's bodies present, several years' duration
25	-----	4 15 4 plus 4 23 1 plus	weak	positive	Developed bilateral buboes W R negative then became pos itive, probably syphilitic
26	-----	4 23 1 plus	positive	negative	Clinically chancreoid
27	-----	4 23 4 plus	weak	positive	Clinically granuloma inguinale Donovan's bodies present
28	-----	4 23 3 plus	negative	positive	Small lesions on cervix Fusospirochetosis
29	-----	4 23 4 plus	positive	negative	Chancreoid Vulval lesion 3 10, bubo 3 15
31	-----	4 23 1 plus	positive	negative	Chancreoid Multiple lesions of vulva, first appeared 4 months ago
32	5 6 negative	5 6 4 plus	positive	negative	Chancreoid Lesions with bubo
33	5 6 negative	5 6 3 plus	positive	?	Chancreoid Penile lesion with bubo, 1 month
34	5 6 negative	5 6 4 plus	positive	positive	Chancreoid Ducrey's bacillus isolated Labial ulcer for 9 12 months
35	5 6 negative	5 6 1 plus	positive	positive	Chancreoid Ducrey's bacillus isolated. Lesion with bubo for 3 weeks
36	5 6 negative	5 6 4 plus	negative	negative	Suspicious Ducrey's bacillus in smears Two labial ulcers for 2 months
37	5 6 negative	5 6 4 plus	negative	positive	Lesion on vulva which healed quickly, probably lympho granuloma venereum



added as a preservative. The erythrocytic stroma gives this antigen a "murky" appearance, but nevertheless, such preparations have yielded entirely satisfactory results in the skin test during the past two years of our observation.

The antigens for use in the complement fixation tests were prepared as above, using the same strains, but with the variation that 5 c.c. of saline per slant were substituted for 10 c.c. These antigens were titrated against hyper-immune rabbit serum, and although fixation was obtained, it was found that no more than two antigenic units could be satisfactorily used because the murkiness interfered with the reading of hemolysis. The rabbits were immunized with killed cultures, grown in rabbit blood.

In the preliminary tests on sera from chancroidal patients, the results were entirely negative. To corroborate this, as many as possible of our clinic chancroidal patients were rounded up. Tests on their sera were also negative. They were also negative in patients who on the basis of a positive skin test had had chancroid in the past (Table I). Later in the course of the studies, an antigen made from growth in rabbit blood was tested. Positive fixations were obtained.

TABLE II

COMPLEMENT FIXATION TESTS USING HEMOLYZED, WASHED RED BLOOD CELL RESIDUE AS ANTIGENS

CASE*	RABBIT CELLS	HUMAN CELLS	REMARKS
1	4 plus	negative	Granuloma inguinale
4	4 plus	negative	Chancroid
24	4 plus	negative	Granuloma inguinale (old chancroid)
27	4 plus	negative	Granuloma inguinale
32	4 plus	negative	Chancroid
33	4 plus	negative	Chancroid
34	4 plus	negative	Chancroid
35	2-3 plus	negative	Chancroid
36	3 plus	negative	Chancroid
37	4 plus	negative	Probably lymphogranuloma venereum

\*Numbers correspond to those in Table I.

An additional number of known sera were tested, and as controls some 82 routine Wassermann sera from the health department. All of the sera from the chancroidal patients gave complete fixation as did 34 of the controls. The clinical histories of the latter were unknown, but from our two-year studies on chancroidal infection, it was felt that this apparent high incidence was altogether out of line. Still, the discrepancy in the two antigens could not be accounted for. Six sera from known chancroidal cases were then tested simultaneously with the two antigens: that made from human blood gave negative fixations, while that made from rabbit blood yielded complete fixation in all but two instances—a one plus and a three plus. A few tests using the rabbit blood antigen were made on ward patients having negative skin reactions and in whom there was no evidence past or present of chancroidal infection. Complete fixations were obtained. It was evident, therefore, that something inherent in the antigens made from growth in rabbit blood was responsible for the positive fixations observed. To substantiate this opinion, "antigens" were prepared using just the washed sediment, appropriately diluted, from laked human and rabbit bloods from amounts of blood that were customarily added to the agar slants when cultures

were grown. These were tested simultaneously with sera from six known chaneroidal cases, one old (?) chaneroid and three granuloma inguinale (Table II). The former "antigen" gave negative reactions, whereas the latter gave all positive. The rabbit "antigen," however, yielded negative results when tested against homologous serum. It is evident, therefore, that *all* the fixations obtained were undoubtedly due to a nonspecific fixation between residue rabbit blood cells and human sera.

#### SUMMARY AND CONCLUSIONS

1 Antigens which were satisfactory in the skin test for chaneroidal infection and prepared from growth in human blood, gave negative results when used in the complement fixation test.

2 Antigens prepared from rabbit blood growth yielded false positive reactions in the complement fixation test, and were due apparently to some inherent quality of the rabbit blood cells.

3 With the antigens available, the complement fixation test was an unsatisfactory procedure in the diagnosis of chaneroidal infection.

The authors wish to thank Dr. Anna D. Dulancy, Department of Bacteriology, University of Tennessee Medical School, for supplying sera and data on several chaneroidal cases.

#### REFERENCES

- 1 Sanderson, E. S., and Greenblatt, R. B. The Cultivation of *Hemophilus ducreyi* and the Preparation of an Antigen for the Intracutaneous Diagnosis of Chaneroidal Infection, *South M. J.* 30: 147, 1937.
- 2 Greenblatt, R. B., and Sanderson, E. S. Chaneroidal Vaccine, *Am. J. Clin. Path.* 7: 193, 1937.
- 3 Greenblatt, R. B., and Sanderson, E. S. The Diagnostic Value of the Intradermal Chaneroidal Test, *Arch. Dermat. & Syph.* 36: 486, 1937.

---

### A MULTIPLE RING MAKER FOR THE KLINE TEST\*

JOHN H. MILLS, M.D., BALTIMORE, MD

---

NUMEROUS devices have been described to facilitate the performance of the Kline test<sup>1, 2</sup>. A rigid brass multiple ring maker has been found to reduce the time required to make the paraffin rings for the Kline diagnostic and exclusion tests.

Cut twelve one inch lengths of brass tubing 16 mm ( $\frac{5}{8}$  inch) outside diameter and about 1.2 mm wall thickness. The tubing should be cut squarely, preferably by a mechanical hack saw. These short brass tubes are soldered to a heavy brass plate ( $\frac{3}{16}$  to  $\frac{1}{8}$  inch thick). This brass plate should be of the same size as the glass slides used in the test. The position in which the brass tubes are soldered to the brass plate should correspond to the desired position of the paraffin rings on the Kline slide, usually three rows of

\*From the University Hospital and College of Medicine, University of Maryland, Baltimore.

four tubes. Space is saved if the rows are somewhat staggered. There must be a space between tubes, so that an excessive amount of paraffin is not held at the point of contact between tubes.

After soldering in place on the brass plate, the free surface of the bank of tubes is dressed with a file till the ends of the tubes are in the same plane. Further grinding to a plane surface is accomplished by rubbing on emery paper which has been glued to plate glass to prevent buckling. Final grinding is accomplished by rubbing the surface of the bank of tubes on plate glass sprinkled with emery powder, being careful not to tip the instrument at any time during the grinding to avoid grinding more off of the edges than the center. When completed, the free ends of the brass tubes should all make contact with a plane surface throughout their entire circumference.

To furnish a vent, a small hole is drilled through the plate in the center of each tube. A heat-resisting handle, such as a bakelite radiator valve handle, is fastened by a screw to the top of the plate to complete the instrument, which when finished resembles a rubber stamp in appearance (Fig. 1).

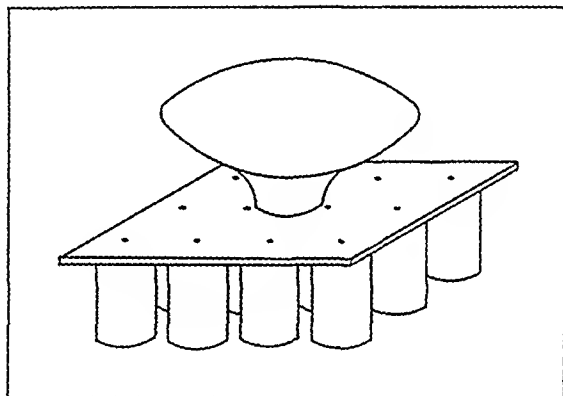


Fig. 1.

It is used in a similar fashion to a rubber stamp by dipping the instrument into melted paraffin (about  $80^{\circ}$  C.), allowing the stamp to become warm, shaking off the excess paraffin, and then transferring to the Kline slide.

The resulting height and width of the paraffin ring is regulated by adjusting the temperature of the paraffin, the temperature of the brass stamp itself, the depth to which the stamp is immersed in the paraffin bath, the temperature of the glass slide to which the transfer is made, and the time intervening between removal of the stamp from the paraffin bath and its application to the slide. Practice will enable the user to determine the most satisfactory conditions for use.

This ring maker possesses the advantage of perfect rigidity. If the surface of the ends of the brass tube becomes marred, it is easily resurfaced by repeating the grinding with emery as above described.

It has also been found useful to control the radius of rotation of the tray containing the Kline slides by the simple method of fitting a small short peg

into the bottom of the tray. This peg fits into a large hole in a board placed on the table or, if desired, into a hole in the laboratory table itself. The radius of the peg, when subtracted from the radius of the hole, will give the radius of rotation. In operation the small peg is made to travel around the circumference of the large hole, thus limiting the radius of rotation. This prevents the otherwise frequent spilling of the contents of the rings.

#### REFERENCES

- 1 Magath, T. B. Notes on Apparatus for Performing the Kline Test, *J. LAB. & CLIN. MED.* 18: 83, 1932.
- 2 Brown, A. S., and Pentler, C. F. An Improved Ring Maker and An Emulsion Pipette for the Kline Test, *J. LAB. & CLIN. MED.* 23: 17, 1937.

## TRIAxIAL CORRELATION OF HEMATOLOGIC INDICES ITS SIGNIFICANCE IN CLASSIFICATION AND TREATMENT OF ANEMIAS\*

KATSUJI KATO, M.D., PH.D., CHICAGO, ILL.

THE importance of hematologic indices in the diagnosis and treatment of anemias is now so well established in clinical practice that even a cursory discussion of the subject may seem superfluous, yet the definite relationship existing between the color, volume, and saturation indices is but vaguely appreciated by the average clinician. The exposition of fundamental principles involved in the application and interpretation of index numbers as given in most hematologic textbooks is unavoidably vitiated by pages of awkward equations that are hardly inviting to the eyes of busy practitioners. To offset this shortcoming, a few authors, particularly Osgood (1927, 1935), have constructed tables and charts whereby the index figures may be obtained at a glance without resorting to tedious though simple arithmetic. That these three indices are, as a matter of fact, mutually interdependent and, therefore, amenable to a simple mathematical correlation by means of a suitable graphic method has never been pointed out even by hematologists. The graphic chart here presented is an attempt to offer a simple, accurate, and practical correlation of the indices, with special reference to classification and treatment of anemias.

Each of the three major hematologic indices of diagnostic importance, the color index (Hayem, 1878), the volume index (Capps, 1903), and the saturation index (Haden, 1923), is expressed in relative numbers, the unit 1.0 representing the value for a normal blood picture. Upon mathematical manipulation of index values under both normal and pathologic conditions, a complete and rather constant relationship is found to exist between them.

\*From the Department of Pediatrics, University of Chicago.  
Received for publication March 16, 1938.

This interrelationship is clearly demonstrable when the indicial values are expressed in their logarithmic equivalents.

Thus, since

$$z = \frac{x}{y},$$

in which  $x$  represents the value for the color index,  $y$  the volume index, and  $z$  the saturation index,

$$\log z = \log x - \log y,$$

therefore,

$$\log x - \log y - \log z = 0.$$

This is an equation of the general form:

$$ax + by + cz = K,$$

in which the three variables are mutually related and can be expressed on the triaxial chart, as Hastings and his co-workers (1931, 1934) have done in

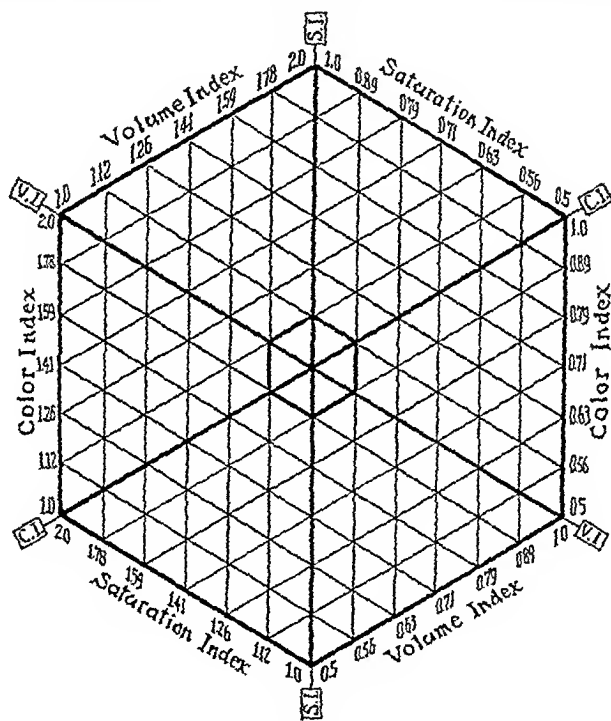


Fig. 1.

their studies on the acid-base balance of the blood under normal and experimental conditions. Hematologic data as converted into index numbers are likewise suitable for plotting on triaxial coordinate papers.

#### CONSTRUCTION OF THE TRIAXIAL CHART

The triaxial chart (Fig. 1) here presented is so constructed, following the general procedure as employed by Hastings and Steinhaus (1931), that the three axial lines connecting the six corners of the hexagon represent the ideal normal values for color index, volume index, and saturation index, respectively, and all meeting at a point in the geometric center. This point,

therefore, represents the uniform value of 10 for all three indices, from which deviations of  $\pm 0.1$  are customarily regarded as being within the normal limits. This range of normal variations is represented by a smaller hexagon enclosed by heavy lines within the center of the chart (see Fig 2). The axis connecting II and VIII o'clock represents the indicial value of 10 for the color index, and the parallel lines, both above and below this axis, denote the varying degrees of gradation as indicated on the left and right margins of the chart, respectively. Likewise, the axial line passing in the direction of IV and X o'clock, indicates the value of 10 for the volume index, with similarly varying graduations both above and below in parallel lines. The normal value of 10 for the saturation index runs in the vertical direction of VI and XII o'clock, with its sets of parallel lines on either side of the main axis.

The marginal figures of the hexagonal chart represent three sets of relative numbers corresponding to the three indices, each set being expressed in exactly comparable values, ranging from 0.5 to 2.0, with 1.0 at the axial

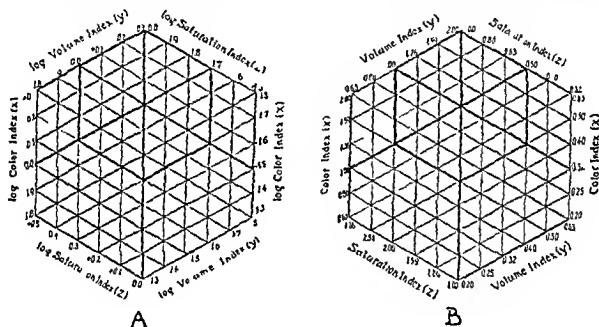


Fig 2

positions. The logarithmic values are placed at regular and evenly spaced intervals on the chart (Fig 2 A), and the indicial numbers correspond each to its own logarithmic equivalent (Fig 2 B). The actual portion of the coordinate field utilized for the present chart is demarcated by heavy lines at the top of the original chart.

Thus, the triaxial chart as applied to indicial numbers is a strictly accurate mathematical expression of the actual interrelationship existing between the three indices. The main advantage of the chart is its convenience in locating the indicial position of a given blood picture, with special reference to its hematologic classification, and in recording the progress of the anemia under therapeutic management, as well as in determining its saturation index. These features distinguish the triaxial coordinate chart here presented from the simple calculation chart of Osgood (1927, 1935).

In the practical use of the chart for hematologic diagnosis the color and volume indices are first calculated from actual determination of red cell count per cubic millimeter of blood, of the amount of hemoglobin in grams per

100 c.c., and of the volume of packed cells in percentage of the whole blood.\* Since the saturation index is a quotient obtained by dividing the color index by the volume index, its graphic position can be automatically located on the chart once the color and volume indices are known. This obviates the necessity of calculating the saturation index separately.

#### NORMAL AND ARBITRARY STANDARDS

The calculation of hematologic indices requires the use of certain standards which may be either actual figures representing average values obtained from observations on normal individuals, or some simple round figures arbitrarily selected for convenience in calculation. Some authors (Osgood, Haskins, and Trotman, 1932) justly emphasize the fact that the standards with which any pathologic blood is to be compared must be actual average values

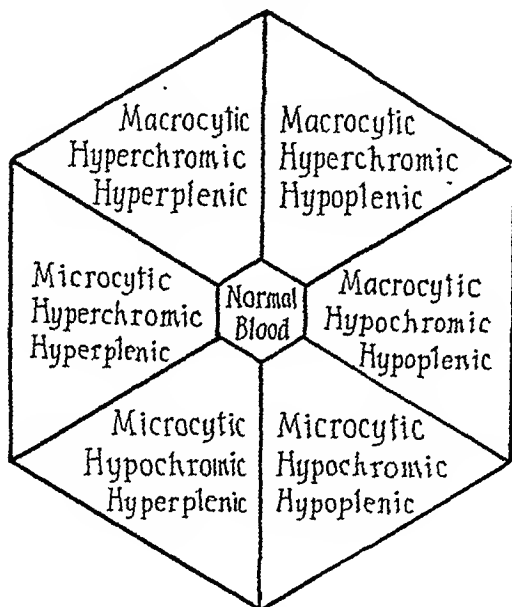


Fig. 3.

obtained from normal individuals of the patient's own age and sex group. This is entirely correct, especially if the comparison is to be made directly in absolute numbers. Such a set of average figures, which at least in certain age groups are available, may be designated as normal standards. It goes without saying that such standards must be derived from the most extensive observations on very large groups of normal individuals.

As a matter of fact, however, in the calculation of hematologic indices actual average figures of normal values are not absolutely necessary, inasmuch as the indicial numbers are always but relative in nature. Any round numbers which are fairly representative of the normal average values and at the same time simple enough for ready calculations can be used for this purpose

\*In working with infants and small animals, in which capillary blood is more readily obtainable than is venous blood, the use of a recently devised microhemopipette (Kato) requiring only 0.05 c.c. of oxalated blood is recommended for the determination of cell volume (*J. LAB. & CLIN. MED.* 23: 380, 1938). If venous blood is available, other types of hematocrit, using larger amounts of blood, are satisfactory. In either case the proportion of oxalate to the blood should be 1:500 (or 1 mg. per 0.5 c.c.).

with complete satisfaction. These standards, called arbitrary standards, may thus be regarded as common factors which, when consistently used throughout all calculations, will yield strictly comparable indices. It is at once clear then that the use of simple arbitrary standards as common factors in indicial equations is preferable to employment of the more complex and consequently more troublesome sets of actual normal standards. The following set of figures, uniformly employed in all calculations are customarily regarded as arbitrary standards, each representing the equivalent of 100 per cent as normal.

Erythrocytes (millions per cmm)	5 00 (Normal range 4 50-5 50)
Hemoglobin (gm per 100 cc)	15 0 (Normal range 14 0-16 0)
Packed cell volume (per 100 cc)	45 0 (Normal range 40 0-48 0)

The above standards are round figures which maintain a rather simple relation to each other, so that the calculation of indices is considerably simplified, as shown in the following paragraph.

#### CALCULATION OF INDICES

Using the set of arbitrary standards as recommended in the preceding paragraph, the calculation of hematologic indices may be obtained by means of the following equations:

$$(1) \text{ Color Index} = \frac{\text{Hemoglobin (grams per cent)}}{\text{Erythrocytes} \times 3}$$

in which the factor 3 has been derived from the ratio that the figure 15 0 (arbitrary standard for hemoglobin) maintains to the figure 5 00 (arbitrary standard for erythrocytes)

$$(2) \text{ Volume Index} = \frac{\text{Packed cell volume (per cent)}}{\text{Erythrocytes} \times 9}$$

where the factor 9 has been obtained from the ratio that the figure 45 0 (arbitrary standard for packed cell volume) maintains to the figure 5 00 (arbitrary standard for erythrocytes)

The corpuscular values of Wintrobe (1932-1933) may also be converted into standard index numbers by use of the following equations:

$$(1) \text{ Color Index} = \frac{\text{Corpuscular hemoglobin (C H)}}{30}$$

in which the denominator 30 is the arbitrary standard for mean corpuscular hemoglobin in average normal individuals, as expressed in micromicrograms

$$(2) \text{ Volume Index} = \frac{\text{Corpuscular volume (C V)}}{90}$$

where the denominator 90 is the arbitrary standard for normal mean corpuscular volume expressed in cubic microns

$$(3) \text{ Saturation Index} = \frac{\text{Corpuscular hemoglobin} \times 3}{\text{Corpuscular volume}}$$



in which the factor 3 represents the ratio that the figure 30 (arbitrary standard for normal mean corpuscular hemoglobin) maintains to the figure 90 (arbitrary standard for normal mean corpuscular volume).

#### CLASSIFICATION OF ANEMIAS BY THE TRIAXIAL CHART

The triaxial chart here presented may also be used for the purpose of accurately placing various types of anemias according to their indicial characteristics. The three axial lines serve as precise boundaries separating the six groups (Fig. 2). In the geometric center of the chart is a small hexagon which represents the limits of variations in normal blood, while the peripheral triangles classify the anemias with all the possible combinations of indicial characteristics. The terms macrocytic and microcytic customarily refer to the dimensional qualities of erythrocytes; hence macrocytic anemias have relatively higher volume index than the microcytic. Hyper- and hypochromic anemias are distinguishable primarily on the basis of color index values. The terms hyper- and hypoplenic, used for the first time as far as the writer is aware, refer to the degree of saturation of erythrocytes with hemoglobin as determined by the saturation index. Incidentally, as noted by Capps (1903), supersaturation of erythrocytes with the pigment very seldom, if ever, occurs. A low saturation index usually suggests an anemia due to chronic blood loss (Osgood, 1926).

The classification of anemias here evolved by means of the triaxial chart offers a definite aid in determining rational and specific therapy for each group. It is now known that macrocytic types of anemia respond favorably to liver and liver substitutes, while iron is the specific for the microcytic types. It is also clear that the anemia characterized by a combination of macrocytosis and hypochromia requires both iron and liver. Anemias with saturation index above 1.1, though extremely rare, respond poorly to any type of treatment, since this group postulates a highly abnormal developmental anomaly in erythropoiesis. It is quite conceivable, however, that it is this latter group of anemias which requires such therapeutic adjuncts as vitamins, iron catalysts, and other marrow stimulants, as well as additional substances directly or indirectly concerned in the formation of erythrocytes and hemoglobin.

The triaxial chart is, furthermore, well adapted for recording the course of progress under treatment. As a rule, the more severe the degree of anemia the more peripheral is the position of the blood picture on the chart. Under specific and effective therapy the indicial position of the blood will move toward the center, thereby indicating its return to the normal.

#### SUMMARY

A triaxial chart correlating the color index, volume index, and saturation index, together with simplified equations for calculation of these indices, is presented. An accurate hematologic classification of all types of anemia, so essential a foundation for rational therapy, is made possible by this chart.

It also affords a simple and convenient method for recording the changes in index value influenced by specific treatment.

## REFERENCES

- 1 Capps, J A A Study of Volume Index Observations Upon the Volume of Erythrocytes in Various Disease Conditions *J M Research* 10 367, 1903
- 2 Haden, R L Accurate Criteria for Differentiating Anemias, *Arch Int Med* 31 245, 1923
- 3 Idem The Technique of Determination of the Relative Mass, the Individual Cell Volume, and the Volume Index of Erythrocytes of Man *J LAB & CLIN MED* 15 736, 1920
- 4 Idem The Technic of a Blood Examination *J LAB & CLIN MED* 17 813, 1932
- 5 Hastings, A B, and Steinhaus, A H A New Chart for the Interrelation of Acid Base Changes and Its Application to Exercise, *Am J Physiol* 96 538, 1931
- 6 Hayem, G Recherches sur l'anatomie normale et pathologique du sang Paris, 1878, Masson et Cie
- 7 Osgood, E E Hemoglobin, Color Index, Saturation Index and Volume Index Standards, *Arch Int Med* 37 685, 1926
- 8 Idem Tables for Calculation of Color Index, Volume Index, and Saturation Index Based on Recently Determined Standards, *J LAB & CLIN MED* 12 899, 1927
- 9 Idem A Textbook of Laboratory Diagnosis, ed 2, Philadelphia, 1935, P Blakiston's Son & Co
- 10 Osgood, E E, Haskins, H D and Trotman, F E The Value of Accurately Determined Color, Volume and Saturation Indexes in Anemias *J LAB & CLIN MED* 17 859, 1932
- 11 Shock, N W, and Hastings, A B Studies of the Acid Base Balance of the Blood VIII Variation in the Acid Base Balance of the Blood in Normal Individuals, *J Biol Chem* 104 585, 1934
- 12 Wintrobe, M M Size and Hemoglobin Content of the Erythrocytes, *J LAB & CLIN MED* 17 899, 1932
- 13 Idem Blood of Normal Men and Women, Erythrocyte Counts, Hemoglobin and Volume of Packed Red Cells of 229 Individuals *Bull Johns Hopkins Hosp* 53 118, 1933
- 14 Idem Macroscopic Examination of Blood, *Am J M Sc* 185 58, 1933

## PHOTOELECTRIC COLORIMETRY\*

## I DETERMINATION OF BLOOD IRON AND HEMOGLOBIN

JEROME E ANDLES, PH D, M D, AND DAVID W NORTHUP, PH D,  
MORGANTOWN, W VA

## INTRODUCTION

THE inaccuracy of the eye in matching the intensity of colors is only too well known. This difficulty is not so marked in the blue purple end of the color scale, but is quite pronounced in the red yellow part. Also, the human eye is subject to the many variations of any organ of the body, the variation being even more accentuated with different individuals. Consequently, eye colorimetry, even in the blue end of the color scale, is far from being all that might be desired, and in the red part is often entirely unsatisfactory.

The photoelectric cell, however (which is essentially an electric eye), does not measure the intensity of colors as such, but measures only the degree of transmission of light, therefore, it reacts similarly to all colors even though it is somewhat more sensitive to the blue and purple shades. It is not subject to the variations of the human eye, having an accuracy characteristic of

\*From the Departments of Pathology and Physiology West Virginia University Medical School Morgantown

Received for publication March 16 1938

electrical instruments in general. Its introduction into the field of colorimetry has, therefore, been an important step, and augurs an era of more accurate chemical analysis in biological work.

In general there are two types of photoelectric colorimeters: those having one and those having two photoelectric cells. The one-cell instruments consist essentially of a photoelectric cell, a source of light of constant intensity (requiring a storage battery for its production), and a cup of standard size for interposing the solution to be tested between the light and the cell. Readings are taken with a set of standard solutions, and a curve plotted; the concentration of the unknown is then obtained by the location of its reading on the prepared graph. The "readings" may simply be the output of the photoelectric cell in amperes with different intensities of light; they may consist of the variation in the amount of light (by a calibrated shutter) or the electrical resistance necessary to keep the output of the cell constant with different color intensities. Such instruments are described by Kesten and Zucker,<sup>1</sup> Sheard and Sanford,<sup>2-4</sup> Ellis,<sup>5</sup> Evelyn,<sup>6</sup> Lebowich,<sup>7</sup> and others. Most of these workers employ the use of radio vacuum tubes and accessory batteries, or the use of galvanometers and other electrical material. The use of filters is advocated by some workers to "blot out" the parts of the spectrum not occupied by the color being measured.

The chief advantages of single-cell instruments are their simplicity in construction, their relatively low cost, and their ease of adaptability to the use of filters. Their disadvantages lie in the need for a constant source of light (furnished only by a battery), and the fact that fatigue of the cell and variations due to temperature changes, directly (and measurably) affect the colorimeter readings.

The two-cell instruments consist essentially of two photoelectric cells, a source of light (falling simultaneously upon both cells), a standard cup for interposing colored solutions, and some arrangement for measuring the changes in the system produced by different intensities of colors. The advantages of this type of apparatus are: first, the source of light need not be absolutely constant (eliminating the storage battery) since variations in the light affect both cells similarly; and second, the readings are taken by balancing the cells against each other by the use of a sensitive galvanometer (null-point method). The null-point method largely eliminates the errors arising from an inconstant light source, temperature changes, and cell fatigue. The instruments can also be adapted to a wider variety of uses, and the readings can be duplicated with different instruments.

Such instruments have been described by Exton<sup>8-10</sup> and Goudsmit and Summerson.<sup>11</sup> In Exton's instruments the two cells are connected to a galvanometer by suitable resistances, and the readings made as the amount of light (determined by the use of a calibrated shutter) necessary to keep the galvanometer reading constant when colored solutions are placed in the cup. The values for known solutions are plotted on a graph against the concentration, and the curve used to make the unknown determinations. In Exton's last instrument<sup>10</sup> the cells are mounted at 180° angles to each other, and the

light source is capable of being moved from one side to the other to produce a null-point reading on the galvanometer. This allows the direct comparison of standards and unknowns. Goudsmit and Summerson's instrument<sup>11</sup> is arranged like an ordinary colorimeter, with the eye being replaced by two photoelectric cells connected by suitable circuits to a galvanometer. The readings are taken by adjusting the depth of the colored solution in each cup until the galvanometer is at 0, indicating that the conduction of light (intensity of color) through each column of liquid is the same.

Although the null-point instruments are more desirable than the one-cell type, the initial cost is larger, and they present more difficulties to the use of filters. Our investigation of photoelectric colorimeters led us, therefore,

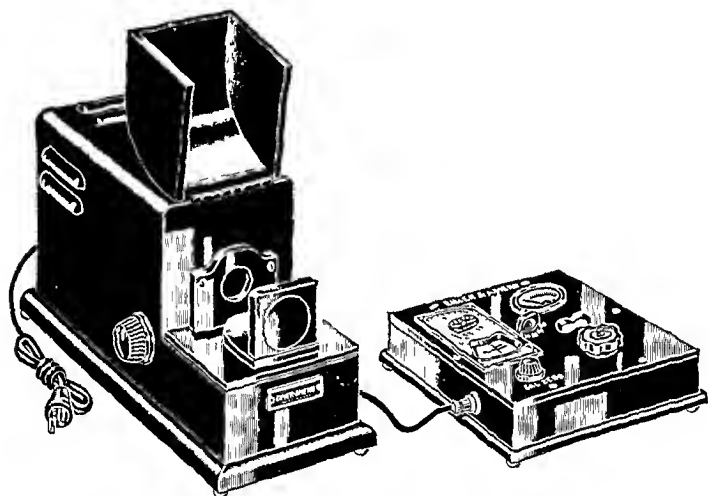


Fig. 1.—Complete photoelectric colorimeter (without voltage regulator).

to purchase an instrument made by the Eimer and Amend Co. of New York\* (see Fig. 1). This apparatus is composed of two photoelectric cells using the same light source, the currents from the cells being balanced by a potentiometer (instead of adjusting the depth of the solution<sup>11</sup> or by a shutter arrangement<sup>8-10</sup>). The most important feature is that the first cell is circular in shape and surrounds the beam of light going to the second cell. In this way *one* filter can be interposed between the light source and both cells, rendering the use of filters a very simple matter. Readings are made directly as per cent conduction of light, by means of a suitable potentiometer (Weston). The cells are selenium on iron, they are quite stable and emit two or three

\*The circuit is simple and the entire apparatus (except the potentiometer) can be easily constructed by anyone wishing to go to such trouble. The instrument made by Eimer and Amend has, however, proved quite satisfactory, and they have generously provided us with much of the information about the instrument that is given in this report.

times the amount of current given by most of the cells in current use. The instrument is also arranged for nephelometry by an adjustment of the second cell. The actual circuit is not given, but it consists essentially of two photoelectric cells connected to a galvanometer by a modified wheatstone bridge arrangement, the variable resistance being calibrated to read per cent conduction of light.

In the operation of the apparatus, the instrument is first allowed to "heat up" for fifteen to twenty minutes (for accurate work) in order to allow the output of the cells to become constant. The output of the first cell is then adjusted to some definite value, and the two cells balanced with water (or a blank of the reagents) in the interposed cup. Then the cup is filled with the unknown solution, and the per cent of conduction observed by again balancing the colorimeter. A graph is first plotted with solutions of known concentrations, and the concentration of the unknowns taken directly from the curve.

Suitable filters abet the determinations of most red, yellow, and green colors. We have used Wratten filters exclusively, and have had occasion to use only numbers 63, 47, and 29. These filters have not shown any measurable change with one year of use.

The use of a voltage regulator is desirable if the line voltage is irregular, especially when filters are employed. The location of the cells at different distances from the source of light causes a slight variation in the output of the cells, with variations in the intensity of the light. When filters are not used, the intensity of the light (as measured by the current output of the circular cell, and whose output *must* be kept constant) can be easily regulated by the rheostat on the instrument itself. If a filter is used, the photoelectric cell output is too small to be read accurately, and accurate values are best obtained by keeping the voltage coming into the machine constant by means of a voltage regulator.

The use of two solution cups may be desirable: one for the blank of reagents (or water) and one for the unknown solutions. However, these cups must be kept extremely clean (soap and water), as invisible traces of pigment dried on the surface of the cups affect the colorimeter readings measurably. This precaution may be neglected if only one cup is used for all solutions.

#### EXPERIMENTAL

In this work we used the photoelectric colorimeter described above. The slot for the use of filters had to be slightly enlarged to allow the use of Wratten filters. A voltage regulator was found essential as our current supply was quite inconstant. Filters were used to "spread" the conductivity readings over more of the scale, and a number 47 Wratten filter (gelatin) was found most suitable for both blood iron and hemoglobin curves.

Hemoglobin was determined by estimating blood iron, using the method and curve below (Fig. 2), and the hemoglobin values plotted against the conduction figures obtained from water dilutions (1:400) of whole blood (same sample). The curve (Fig. 3) was then drawn through the points plotted. The actual procedures in making the determinations are given below.

**Method for Blood Iron—Principle** This is the method of Wong<sup>1</sup>—adapted to the photoelectric colorimeter. The iron in the blood is liberated with concentrated sulfuric acid, oxidized to the ferric state with potassium persulfate, and the color developed with potassium thiocyanate. The curve (Fig. 2) was prepared by substituting standard solutions of pure ferric iron for the blood filtrate, and plotting the values.

**Procedure** Introduce 1 cc of well mixed oxalated blood into a 100 cc volumetric flask, add 4 cc of concentrated  $H_2SO_4$  (shaking well all the time), and allow to stand at least one to two minutes. Add 4 cc of a saturated solution of potassium persulfate ( $K_2S_2O_8$ )<sup>\*</sup> and shake. Cool under the cold water

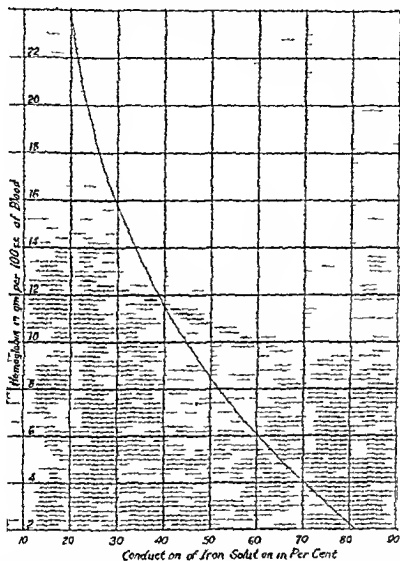


Fig. 2—The light conduction of ferric thiocyanate solutions. The conduction of ferric thiocyanate solutions of varying concentrations has been plotted against the equivalent hemoglobin value (as calculated from the known iron content).

tap, and add (cautiously to prevent foaming) distilled water to within 5 to 10 cc of the mark. Then add 4 cc of 10 per cent  $NaOH$ , dilute to the mark, and mix well.

Filter through non free filter paper (Whatman No. 40 is satisfactory). Transfer 20 cc of this filtrate† to a 25 cc volumetric flask, add 1 cc of potassium persulfate, and dilute to the mark with approximately 3 N potassium thiocyanate‡. Mix well.

\*Prepared by adding about 4 gm of pure potassium persulfate to 100 cc of warm distilled water, shaking well and allowing the excess salt to settle out.

†If standard iron solutions are used the procedure is started at this point.

‡Prepared by dissolving 291 gm of pure  $KCN$  in distilled water and making the volume up to 1000 cc. Filter if not entirely clear.

The colorimeter should have been turned on about fifteen to twenty minutes before starting the experiment to allow it to "heat up." Fill the sample cup with pure water, adjust the line voltage to some convenient figure (112) by means of a voltage regulator, and then set the current coming from the instrument at 0.40 milliamperes (the latter is imperative). Now insert a No. 47 Wratten filter (2 inches square), and set the instrument at 100. Balance the two cells, keeping the entering voltage at 112 volts.\*

Now fill the cup (it is convenient to have two cups, one for the blank and one for the unknown sample) with the sample prepared above, and determine the percentage of conduction. By using the curve in Fig. 2, the hemoglobin is read off directly, and the blood iron is found by multiplying by 0.00335.

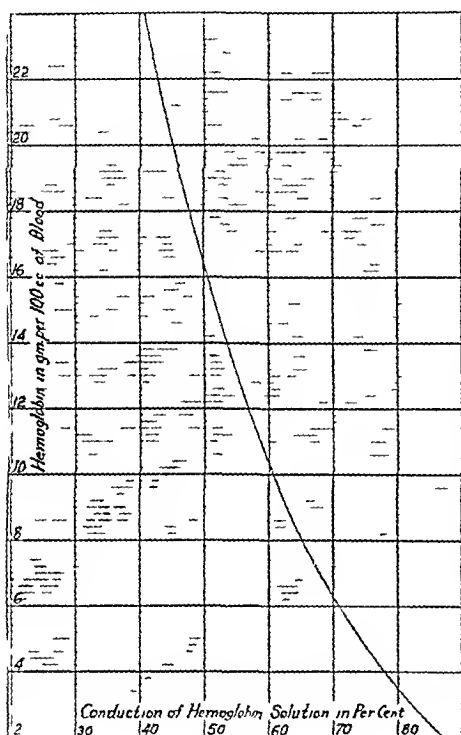


Fig. 3.

Fig. 3.—The light conduction of hemoglobin solutions. The conduction of whole blood diluted 1:400 with distilled water is plotted against the actual hemoglobin concentration.

Fig. 4.—Comparison of the conduction curves of different concentrations of hemoglobin without a filter and with Wratten filters 74 and 47.

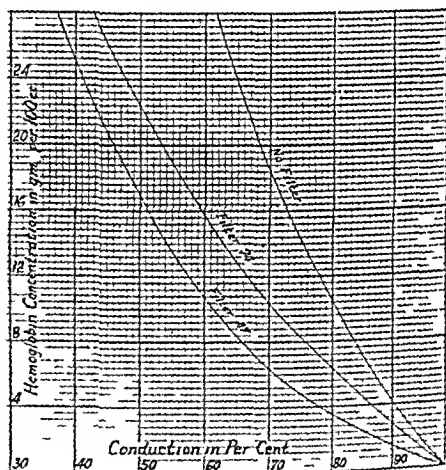


Fig. 4.

**Direct Method for Hemoglobin.—Procedure.** Using a 0.05 c.c. pipette (graduated to contain),† transfer 0.05 c.c. of oxalated or finger-tip blood to exactly 20 c.c. of distilled water,‡ and wash the pipette with the solution

\*When filters are not used, the current in the colorimeter can be kept at 0.40 milliamperes by the rheostat on the machine. However, when a filter is employed, the current generated by the photoelectric cells is too small to accurately measure, and a constant cell output is best maintained by first adjusting the current from the electric cell to some value (0.40 millamp.), noting the voltage, and keeping the entering voltage at this figure all through the determination.

†We obtained such pipettes from the Fisher Scientific Co. A larger pipette can be used just so the dilution is 1:400. Care must be taken not to draw the blood very far past the 0.05 c.c. mark, as the blood sticking to the side of the pipette introduces an appreciable error.

‡Sheard and Sanford<sup>2</sup> diluted the blood 1:200 with 0.1 per cent  $\text{Na}_2\text{CO}_3$  to prevent precipitation of proteins. We have found a dilution of 1:400 to give more consistent and reproducible results. At this dilution the slight haziness of any precipitated proteins is negligible, and a special solution for dilution seems to be unnecessary.

three or four times by sucking it up into the pipette. Do not blow through the solution. This makes a dilution of 1:400. Allow to stand one to two minutes (it may stand four hours) and read the conduction in the photoelectric colorimeter. The instrument is previously set at 100 with water in the cup, using a No. 47 filter as before. The current from the cells is kept at 0.40 milliamperes (without the filter) by adjusting the entering line voltage to the correct amount. From Fig. 3 the hemoglobin is obtained directly. The whole procedure takes about five minutes.

*Types of Filters*—The Wratten filter recommended by Kennedy<sup>13</sup> for hemoglobin measurements (using a direct dilution of blood) is No. 74—a green filter. Experimentation, however, showed the blue No. 47 filter to be distinctly better. In Fig. 4 is shown a comparison between the transmission of plain diluted hemoglobin and that when Nos. 74 and 47 filters are used. It is obvious that a larger amount of the scale of absorption is used with the blue filter. Another point in favor of the 47 filter is that it conducts much more light, and is, therefore, more sensitive to changes in conduction, giving more accurate readings. In fact, we have found this filter to be best in nearly every determination where red or reddish colors are to be matched.

TABLE I  
COMPARISON OF HEMOGLOBIN VALUES BY THE THREE METHODS

SAMPLE	HEMOGLOBIN BY BLOOD IRON METHOD IN GM PER 100 CC	PHOTOELECTRIC METHOD FOR HEMOGLOBIN		SARITZELLIG METHOD FOR HEMOGLOBIN	
		VALUE IN CM %	DIFFER IN GM %	VALUE IN CM %	DIFFER IN GM %
1	17.8	18.0	+0.2	18.5	+1.7
2	15.4	15.6	+0.2	16.3	+0.9
3	15.0	15.0	0.0	14.6	-0.4
4	15.4	15.0	-0.4	15.2	-0.2
5	13.4	12.2	-1.2	13.6	+0.2
6	17.0	17.0	0.0	17.0	0.0
7	15.5	16.2	+0.7	16.7	+1.2
8	17.5	18.0	+0.5	17.8	+0.3
9	16.4	16.2	-0.2	17.2	+0.8
10	17.2	17.5	+0.3	16.6	-0.6
11	15.3	15.4	+0.1	15.8	+0.5
12	14.7	14.2	-0.5	15.2	+0.5
13	16.6	16.2	-0.4	16.8	+0.2
14	16.6	16.2	-0.4	16.2	-0.4
15	15.2	14.8	-0.4	15.0	-0.2
16	15.5	15.4	-0.1	15.2	-0.3
17	5.3	5.2	-0.1	5.4	+0.1
18	12.2	12.0	-0.2	12.6	+0.4
19	13.4	12.3	-1.1	14.1	+0.7
20	13.5	12.6	-0.9	13.4	-0.1
21	15.4	15.1	-0.3	14.1	-1.3
22	15.9	16.6	+0.7	15.0	-0.9
23	14.8	14.4	-0.4	14.0	-0.8
24	14.4	14.0	-0.4	12.0	-2.4
25	11.1	11.2	+0.1	12.2	+1.1
26	14.2	15.0	+0.8	14.6	+0.4
27	13.9	14.0	+0.1	14.0	+0.1
28	6.4	6.6	+0.2	6.5	+0.1
Average	14.5	14.5	0.36*	14.5	0.81*

\*These figures were arrived at by squaring each error and taking the square root of the average of the squared errors. We have referred to this value as the "average error."

*Comparison of Hemoglobin Values*—In Table I is given for comparison the hemoglobin of 28 normal and pathologic subjects using (a) the blood iron



method (as standard of reference), (b) the direct photoelectric method just described, and (c) the Sahli-Hellig hemoglobinometer (prism type). The blood iron determinations were made in duplicate, and the Sahli determinations carried out by one very skilled in handling the instrument. The mean values for all, together with the "average error" are given at the end of the table.

#### DISCUSSION OF RESULTS

It can be seen by the table that the agreement of all three of the hemoglobin methods is quite close. Part of the reason for the Sahli-Hellig values following those for the blood iron so well was due to the determinations being made by one very skillful in handling the instrument. In the hands of the average individual (and in our own hands), such excellent agreement is not obtained. Notwithstanding the close correlation of the Sahli-Hellig and blood iron figures, the "average error"\* (blood iron values taken as a standard of reference) is more than twice as large with the Sahli-Hellig as with the direct photoelectric method. In other words, the photoelectric method is considerably more accurate. The Newcomber disc method was tried in a few cases, but since the values did not check those for the blood iron as well as those from the Sahli, the values are not included.

The advantages of the direct photoelectric method are chiefly as follows: (1) It is very rapid. For a single determination it takes as much time as the Sahli type of instrument, but with a series of determinations it saves considerable time. (2) The readings need not be made for three to four hours after the dilution with water, and they can be made at once. The blood itself can stand at least two or three hours with no appreciable change in the hemoglobin values. (3) Errors arising from waiting too long or too short a time (and such are present in all rapid acid hematin methods) are entirely eliminated. (4) The values are reproducible—to the third significant figure with accurate measurement of the blood and handling of the instrument. Of course, it is obvious that such an expensive piece of apparatus could not be purchased for the determination of hemoglobin alone, but previous work (and the rest of the papers of this series) shows the advantages of using a photoelectric colorimeter in nearly all pathologic chemistry methods.

A point that immediately arises is whether or not the curve given for hemoglobin (Fig. 3) could be used by another operator on another instrument with another filter, or would each laboratory worker have to construct his own curve. It is true that the latter method would undoubtedly be safest and best, but since it occupies considerable time it is our belief that such effort may be unnecessary. While we have not had an opportunity to try this curve on another instrument, the fact that the null-point method of making the readings is employed makes it almost certain that there will be little difference between similar instruments.† Two different filters (same

\*The "average error" was found by taking the square root of the average of the squares of the individual errors. This error (figure) actually means that in two-thirds of all determinations made, the actual error in grams will be less than this amount.

†Exton<sup>9</sup> states that the plotted curve can be used with different instruments (his own type), with an error of less than 5 per cent. We believe this will also hold true with the instrument that we used.

number), gave the same values, likewise different cups (these cups are guaranteed by the manufacturer to be within 0.005 cm of 1.0 cm in thickness, making the greatest possible error less than 0.5 per cent). Of course some practice is necessary to make the most accurate readings, but as soon as one becomes familiar with the instrument he is soon impressed with the ease of manipulation and high degree of accuracy in the duplication of conductivity readings. One thing is certain a fairly accurate curve for hemoglobin can be constructed by each individual in a fairly short time, in the same manner that we made ours.\*

In regard to the blood non method, considerable experimental work showed us that the use of a curve (Fig. 2) gives much more accurate results than the use of simultaneous standards. In fact, much of our earlier work was discarded for the reason that the non was determined by simultaneous comparison with standard solutions. It is certainly a much more rapid procedure, and always gives results that check within the limits of the accuracy of the measurements. We have found Wong's method quite satisfactory, but we believe that some of the newer methods (notably that of Coombs<sup>14</sup> or of Niederhausen and Ferrami<sup>15</sup>) may have additional advantages and even be more accurate.

#### SUMMARY

1 A rapid method for the determination of hemoglobin is described, whereby the light conduction of a watery solution of whole blood is determined with a photoelectric colorimeter, and the concentration of hemoglobin is read directly from a graph. The method seems to be more accurate than the usual acid hematin methods, and is much more rapid where a large number of samples are being analyzed.

2 The determination of blood non by the use of the photoelectric colorimeter is also outlined, and a curve given to obviate the use of standard solutions and to increase the accuracy of the method.

3 A No. 47 Wratten filter was found to be most useful in both hemoglobin and blood non determinations.

We wish to take this opportunity to thank Mr. Fabry L. Hawk for making the Sahli-Hellig hemoglobin determinations that are given in this work.

#### REFERENCES

- 1 Kesten, H. D., and Zucker, T. F. The Determination of the Rate of Hemolysis by the Measurement of Light Transmission, *Am. J. Physiol.* 87: 263, 1928.
- 2 Sheard, C., and Sanford, A. H. Photoelectrometer With One Stage of Amplification, *J. A. M. A.* 93: 1951, 1929.
- 3 Sheard, C., and Sanford, A. H. A Photoelectric Hemoglobinometer, *J. Lab. & Clin. Med.* 14: 558, 1929.
- 4 Sanford, A. H., and Sheard, C. The Determination of Hemoglobin With the Photoelectrometer, *J. Lab. & Clin. Med.* 15: 483, 1930.
- 5 Ellis, M. M. A Photoelectric Apparatus for Turbidity and Light Penetration Measurement, *Science* 80: 37, 1934.

\*The construction of the curve in Fig. 3 is carried out in this manner. The blood iron is determined on several samples of blood. Then each sample is subjected to a number of simple watery dilutions ranging from 1:300 to 1:2000 and the conduction of light through each dilution determined. The 1:400 dilution is taken to equal the concentration of hemoglobin shown by the blood iron measurement (for example 16 gm.). Then the 1:800 dilution would be 8 gm., the 1:600 12 gm., etc. Plotting the dilutions of three or four bloods is sufficient to construct a satisfactory curve.

sample is accurately mixed with an equal volume of the original test meal, and the per cent of phenol red determined on the mixed sample; by a simple correction the per cent present in the original gastric sample can then be calculated.<sup>1</sup> When this is done, the per cent of phenol red in the mixed sample can never be below 50 per cent. This method was carefully checked and found to be very satisfactory.

It was also found that when the gastric samples contained large amounts of bile, it was not always completely removed by the treatment with sodium tungstate and sulfuric acid, so that after alkalinization the gastric samples would have a definite yellow tinge when compared in a colorimeter with a sample of the original test meal. In order to correct for this, a few small crystals of picric acid are added to about 2 c.c. of the test meal standard, and a few drops of the picrated standard mixed in the colorimeter cup with the untreated standard, until the yellow tinge of the standard matches that of the gastric sample. A series of carefully controlled experiments showed that this procedure is entirely satisfactory.

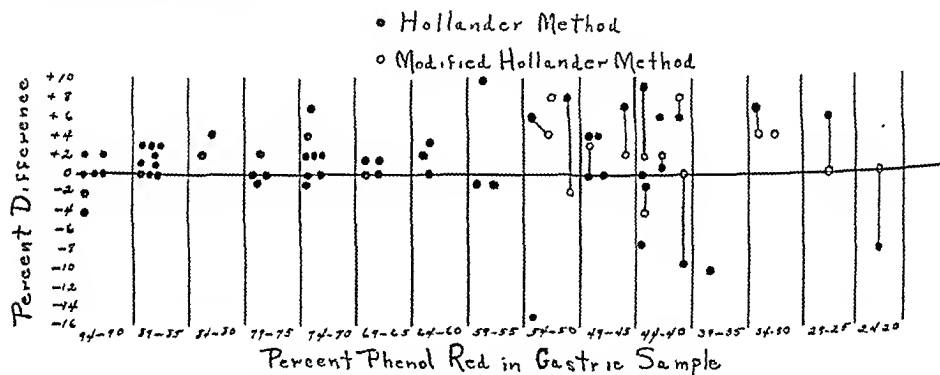


Fig. 1.—The gastric samples were grouped according to per cent present. The line represents perfect agreement between the two methods, and the circles the percentage difference when the modified method was used, and the dots the percentage difference when the Hollander method was used.

Recently Hollander, Penner, and Saltzman<sup>4</sup> have introduced another method for determining the per cent of phenol red in gastric samples. Organic material is precipitated by zinc hydroxide and the samples alkalinized, and the excess zinc hydroxide removed with sodium phosphate. In their method no correction is made for excessive dilution of the samples. They state that all bile is removed, so that no correction is necessary for a yellow tinge of the gastric samples.

In order to compare the two methods, we have performed a series of twenty fractional gastric analyses comprising 65 half-hour gastric samples in which the per cent of phenol red was determined in each gastric sample by both methods. The 2 per cent Liebig's extract test meal<sup>1</sup> was used in all. Thirteen experiments were performed on normal dogs, three on dogs in which we had produced an experimental hypersecretion of acid, and four were on a dog having a gastroduodenostomy, so that large amounts of bile-stained duodenal secretions entered the stomach.

The results are shown in Fig 1. It is seen that down to approximately 50 per cent of phenol red in the gastric samples, the agreement between these two methods is usually quite satisfactory, especially when it is remembered that a variation of  $\pm 2$  per cent is the maximum degree of accuracy possible (in our hands) in matching the phenol red color. Below approximately 50 per cent, the agreement between the two methods is not satisfactory. This is due primarily to the fact that accurate colorimetric determinations cannot be made when the standard solution is two times or more as strong as the unknown solution. Theoretically, according to Beer's law, there should be a direct proportionality between the per cent of phenol red in the sample and the colorimeter reading through all ranges of dilution, practically, however, this is seldom the case. Folin<sup>5</sup> emphasized that accurate colorimetric readings cannot be made when one solution is more than 15 times as strong as the other, a fact which is generally accepted by most investigators.<sup>6, 7</sup> Some of the samples prepared according to the Hollander method were so excessively dilute that it was impossible to make a comparison with the standard. Dilution of the standard with distilled water, so that it closely approximates the unknown is unsatisfactory, because the color of both is then too faint for accurate matching.

When the Hollander method was modified by mixing the very dilute gastric samples with an equal volume of the original test meal, the agreement between the two methods was improved in 8 out of 12 samples. The importance of correcting for excessive dilution is clearly shown by the fact that when no correction was made in the Hollander method, 76 per cent of the samples agreed within  $\pm 1$  per cent by the two methods, but when the correction was made in the Hollander method, 88 per cent of the samples agreed within  $\pm 1$  per cent.

When the dog with a gastroduodenostomy was studied, it was found that the Hollander method often failed to completely remove the bile so that the alkalinized gastric samples had a definite yellow tinge when compared with the standard test meal sample. In order to have made a satisfactory colorimetric reading, it would have been necessary to apply the picric acid correction to the standard solution. During the course of these studies it was again demonstrated that the picric acid correction gives highly satisfactory results.

#### SUMMARY

1 A comparison of the Hollander and Wilhelmj methods for determining the per cent of phenol red in gastric contents shows that down to approximately 50 per cent of phenol red in the gastric samples, the agreement between the two methods is usually satisfactory. Below approximately 50 per cent discrepancies are frequent and marked. When the Hollander method is modified by correcting for excessive dilution of the gastric samples, the agreement is greatly improved.

2 Both methods may at times fail to entirely remove the bile from gastric samples containing excessive amounts, and it may be necessary to apply the picric acid correction.

3 When the Hollander method is modified to correct for excessive dilution of the samples, the two methods are about equally reliable.

method (as standard of reference), (b) the direct photoelectric method just described, and (c) the Sahli-Hellig hemoglobinometer (prism type). The blood iron determinations were made in duplicate, and the Sahli determinations carried out by one very skilled in handling the instrument. The mean values for all, together with the "average error" are given at the end of the table.

#### DISCUSSION OF RESULTS

It can be seen by the table that the agreement of all three of the hemoglobin methods is quite close. Part of the reason for the Sahli-Hellig values following those for the blood iron so well was due to the determinations being made by one very skillful in handling the instrument. In the hands of the average individual (and in our own hands), such excellent agreement is not obtained. Notwithstanding the close correlation of the Sahli-Hellig and blood iron figures, the "average error" (blood iron values taken as a standard of reference) is more than twice as large with the Sahli-Hellig as with the direct photoelectric method. In other words, the photoelectric method is considerably more accurate. The Newcomber disc method was tried in a few cases, but since the values did not check those for the blood iron as well as those from the Sahli, the values are not included.

The advantages of the direct photoelectric method are chiefly as follows: (1) It is very rapid. For a single determination it takes as much time as the Sahli type of instrument, but with a series of determinations it saves considerable time. (2) The readings need not be made for three to four hours after the dilution with water, and they can be made at once. The blood itself can stand at least two or three hours with no appreciable change in the hemoglobin values. (3) Errors arising from waiting too long or too short a time (and such are present in all rapid acid hematin methods) are entirely eliminated. (4) The values are reproducible—to the third significant figure with accurate measurement of the blood and handling of the instrument. Of course, it is obvious that such an expensive piece of apparatus could not be purchased for the determination of hemoglobin alone, but previous work (and the rest of the papers of this series) shows the advantages of using a photoelectric colorimeter in nearly all pathologic chemistry methods.

A point that immediately arises is whether or not the curve given for hemoglobin (Fig. 3) could be used by another operator on another instrument with another filter, or would each laboratory worker have to construct his own curve. It is true that the latter method would undoubtedly be safest and best, but since it occupies considerable time it is our belief that such effort may be unnecessary. While we have not had an opportunity to try this curve on another instrument, the fact that the null-point method of making the readings is employed makes it almost certain that there will be little difference between similar instruments.† Two different filters (same

\*The "average error" was found by taking the square root of the average of the squares of the individual errors. This error (figure) actually means that in two-thirds of all determinations made, the actual error in grams will be less than this amount.

†Exton<sup>3</sup> states that the plotted curve can be used with different instruments (his own type), with an error of less than 5 per cent. We believe this will also hold true with the instrument that we used.

number), gave the same values, likewise different cups (these cups are guaranteed by the manufacturer to be within 0.005 cm of 1.0 cm in thickness, making the greatest possible error less than 0.5 per cent). Of course some practice is necessary to make the most accurate readings, but as soon as one becomes familiar with the instrument he is soon impressed with the ease of manipulation and high degree of accuracy in the duplication of conductivity readings. One thing is certain a fairly accurate curve for hemoglobin can be constructed by each individual in a fairly short time, in the same manner that we made ours.\*

In regard to the blood iron method considerable experimental work showed us that the use of a curve (Fig. 2) gives much more accurate results than the use of simultaneous standards. In fact much of our earlier work was discarded for the reason that the iron was determined by simultaneous comparison with standard solutions. It is certainly a much more rapid procedure, and always gives results that check within the limits of the accuracy of the measurements. We have found Wong's method quite satisfactory, but we believe that some of the newer methods (notably that of Coombs<sup>14</sup> or of Niederhausen and Ferrum<sup>1</sup>) may have additional advantages and even be more accurate.

#### SUMMARY

1 A rapid method for the determination of hemoglobin is described, whereby the light conduction of a watery solution of whole blood is determined with a photoelectric colorimeter, and the concentration of hemoglobin is read directly from a graph. The method seems to be more accurate than the usual acid hematin methods, and is much more rapid where a large number of samples are being analyzed.

2 The determination of blood iron by the use of the photoelectric colorimeter is also outlined, and a curve given to obviate the use of standard solutions and to increase the accuracy of the method.

3 A No. 47 Wratten filter was found to be most useful in both hemoglobin and blood iron determinations.

We wish to take this opportunity to thank Mr. Fabry L. Hawk for making the Sahli Hellig hemoglobin determinations that are given in this work.

#### REFERENCES

- 1 Kesten, H. D., and Zucker, T. F. The Determination of the Rate of Hemolysis by the Measurement of Light Transmission, *Am. J. Physiol.* 87: 263, 1928.
- 2 Sheard, C., and Sanford, A. H. Photoelectrometer With One Stage of Amplification, *J. A. M. A.* 93: 1951, 1929.
- 3 Sheard, C., and Sanford, A. H. A Photoelectric Hemoglobinometer, *J. Lab. & Clin. Med.* 14: 558, 1929.
- 4 Sanford, A. H., and Sheard, C. The Determination of Hemoglobin With the Photoelectrometer, *J. Lab. & Clin. Med.* 15: 483, 1930.
- 5 Ellis, M. M. A Photoelectric Apparatus for Turbidity and Light Penetration Measurement, *Science* 80: 37, 1934.

\*The construction of the curve in Fig. 3 is carried out in this manner. The blood iron is determined on several samples of blood. Then each sample is subjected to a number of simple watery dilutions ranging from 1:300 to 1:2000 and the conduction of light through each dilution determined. The 1:400 dilution is taken to equal the concentration of hemoglobin shown by the blood iron measurement (for example 16 gm.). Then the 1:800 dilution would be 8 gm., the 1:600 12 gm., etc. Plotting the dilutions of three or four bloods is sufficient to construct a satisfactory curve.

6. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, *J. Biol. Chem.* 115: 63, 1936.
7. Lebowich, J.: Simple, Direct-Reading Photoelectric Colorimeter, *J. LAB. & CLIN. MED.* 23: 284, 1937.
8. Exton, W. G.: The Present Status of Clinical Laboratory Measurements With a Note on the Photoelectric Effect, *Am. J. Clin. Path.* 1: 237, 1931.
9. Exton, W. G.: The Photoelectric Scopometer, *Am. J. Clin. Path.* 2: 411, 1932.
10. Exton, W. G.: The Universal Electroscopometer, *Am. J. Clin. Path.* 7: 42, 1937.
11. Goudsmit, A., and Summerson, W. H.: A Variable Layer Photoelectric Comparison Photometer, *J. Biol. Chem.* 111: 421, 1935.
12. Wong, S. Y.: Colorimetric Determination of Iron and Hemoglobin in Blood, *J. Biol. Chem.* 77: 409, 1928.
13. Kennedy, R. P.: The Use of Light Filters in Colorimetry With a Method for the Estimation of Hemoglobin, *Am. J. Physiol.* 78: 56, 1926.
14. Coombs, H. I.: Studies of the Hemoglobin and Iron of the Blood. I. The Determination of Total Iron of the Blood, *Biochem. J.* 30: 1588, 1936.
15. Niederhausern, A. De, and Ferrarini, E.: Photometric Determination of Iron in Blood and Tissues, *Boll. soc. ital.* 12: 229, 1937.

---

## THE DETECTION OF ACETONE AND ACETOACETIC ACID IN URINE\*

JONAS KAMLET, BROOKLYN, N. Y.

---

NUMEROUS qualitative tests (Lange, Le Noble, Rothera, Rantzman) have been proposed for the detection of acetone and acetoacetic acid in urine based on the reaction of these compounds with sodium nitroprusside in the presence of ammonia to form a deep permanganate-violet color. When performed directly on urine, a red color is given by creatinine, which disappears on the addition of acetic acid. Thus, three steps are involved in a direct qualitative determination of acetonuria: (a) addition of nitroprusside solution, (b) acidification (to avoid a false positive due to creatinine), and (c) alkalization with ammonia.

In laboratories where determinations of acetone bodies are performed routinely on all urines, the use of strong aqua ammonia is extremely undesirable. Not only are its fumes highly noisome and noxious to the technician, but the danger of contamination of blood reagents and staining solutions by the volatile ammonia gas is ever present.

Our new method for the detection of acetone and acetoacetic acid is based on the same sensitive and specific color reaction with nitroprusside. However, instead of ammonia, a strongly alkaline organic amine, monoethanolamine, is used. The procedure is quite simple:

Place 10 c.c. of unfiltered urine in a test tube, add one drop (0.05 c.c.) of a saturated aqueous solution of sodium nitroprusside, 1 c.c. of a 15 per cent aqueous solution of monoethanolamine, and mix. In the presence of acetone or acetoacetic acid, the entire contents of the tube will turn to a deep permanganate-violet color within sixty seconds. With a little experience, the

---

\*From the Department of Laboratories, Israel Zion Hospital, Brooklyn.  
Received for publication, March 21, 1938.

technician will soon be able to estimate the concentration of acetone bodies semi quantitatively by the intensity of color (as, for instance trace, one plus, two plus, etc )

This procedure will detect 0.054 per cent of acetone in urine and 0.040 per cent of acetone in aqueous solution within one minute. The normal range of creatinine concentrations in urine (between 0.05 per cent and 0.25 per cent) does not give any color by this method while a saturated solution of creatinine in urine gives a brownish orange color which can hardly be mistaken for the deep permanganate violet color given by acetone and acetoacetic acid.

Acetone determinations were performed by this method on a series of 5000 urines (among which were 521 positive acetones) and compared with the Le Noble reaction simultaneously performed. In every case absolute qualitative agreement between the two procedures was observed.

---

## A COMPARISON OF METHODS FOR THE DETERMINATION OF PHENOL RED IN GASTRIC CONTENTS\*

---

C. M. WILHELMJ, M.D., AND D. E. BACA, B.S., OMAHA, NEB.

---

IN THE usual methods of gastric analysis it is not possible to determine how much of the fluid of the test meal remains in the stomach to dilute the acid secreted by the stomach, nor is it possible to determine the acidity of the total secretions entering the stomach. Wilhelmj, O'Brien, and Hill<sup>1, 2</sup> pointed out these and other deficiencies of the commonly used test meals, and introduced an improved test meal consisting of a specially prepared 2 per cent Liebig's meat extract solution containing 15 mg of phenol red per liter. When the per cent of phenol red is determined in each gastric sample, it is possible to say just how much of the sample consists of secretions mixed with the meal and how much is fluid of the original test meal. It is also possible to determine the acidity of the total secretions entering the stomach.

In the method of Wilhelmj, O'Brien, and Hill, the per cent of phenol red in each gastric sample is determined colorimetrically by comparing the gastric samples with a sample of the original test meal similarly treated. In order to remove organic material which would interfere with the colorimetric determination, equal volumes of the original test meal and of each of the gastric samples are treated with 20 per cent sodium tungstate and 1.33 normal sulfuric acid, centrifuged and alkalinized.

When the fractional method of gastric analysis is used, it is usually found that the final samples are so dilute that it is impossible to make an accurate colorimetric determination of the per cent of phenol red present. In order to correct for the excessive dilution of the final samples, an aliquot fraction of the

---

\*From the Departments of Physiology and Experimental Surgery Creighton University School of Medicine Omaha

Received for publication March 23 1938



sample is accurately mixed with an equal volume of the original test meal, and the per cent of phenol red determined on the mixed sample; by a simple correction the per cent present in the original gastric sample can then be calculated.<sup>1</sup> When this is done, the per cent of phenol red in the mixed sample can never be below 50 per cent. This method was carefully checked and found to be very satisfactory.

It was also found that when the gastric samples contained large amounts of bile, it was not always completely removed by the treatment with sodium tungstate and sulfuric acid, so that after alkalization the gastric samples would have a definite yellow tinge when compared in a colorimeter with a sample of the original test meal. In order to correct for this, a few small crystals of picric acid are added to about 2 c.c. of the test meal standard, and a few drops of the picric acid standard mixed in the colorimeter cup with the untreated standard, until the yellow tinge of the standard matches that of the gastric sample. A series of carefully controlled experiments showed that this procedure is entirely satisfactory.

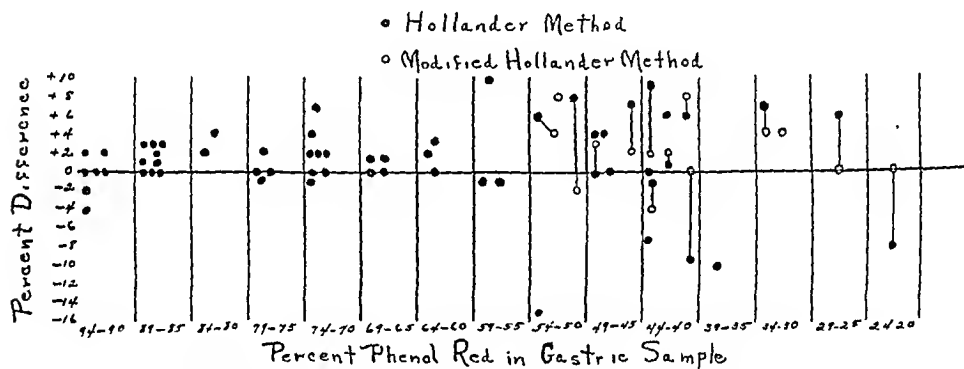


Fig. 1.—The gastric samples were grouped according to decreasing percentages of phenol red present. The line represents perfect agreement between the two methods. The solid dots show the percentage difference between the two methods when the original Hollander method was used, and the circles the percentage difference when the Hollander method was modified by correcting for excessive dilution of the gastric samples.

Recently Hollander, Penner, and Saltzman<sup>4</sup> have introduced another method for determining the per cent of phenol red in gastric samples. Organic material is precipitated by zinc hydroxide and the samples alkalized, and the excess zinc hydroxide removed with sodium phosphate. In their method no correction is made for excessive dilution of the samples. They state that all bile is removed, so that no correction is necessary for a yellow tinge of the gastric samples.

In order to compare the two methods, we have performed a series of twenty fractional gastric analyses comprising 65 half-hour gastric samples in which the per cent of phenol red was determined in each gastric sample by both methods. The 2 per cent Liebig's extract test meal<sup>1</sup> was used in all. Thirteen experiments were performed on normal dogs, three on dogs in which we had produced an experimental hypersecretion of acid, and four were on a dog having a gastroduodenostomy, so that large amounts of bile-stained duodenal secretions entered the stomach.

The results are shown in Fig 1. It is seen that down to approximately 50 per cent of phenol red in the gastric samples, the agreement between these two methods is usually quite satisfactory, especially when it is remembered that a variation of  $\pm 2$  per cent is the maximum degree of accuracy possible (in our hands) in matching the phenol red color. Below approximately 50 per cent, the agreement between the two methods is not satisfactory. This is due primarily to the fact that accurate colorimetric determinations cannot be made when the standard solution is two times or more as strong as the unknown solution. Theoretically, according to Beer's law, there should be a direct proportionality between the per cent of phenol red in the sample and the colorimeter reading through all ranges of dilution, practically, however, this is seldom the case. John<sup>5</sup> emphasized that accurate colorimetric readings cannot be made when one solution is more than 15 times as strong as the other, a fact which is generally accepted by most investigators.<sup>6,8</sup> Some of the samples prepared according to the Hollander method were so excessively dilute that it was impossible to make a comparison with the standard. Dilution of the standard with distilled water, so that it closely approximates the unknown, is unsatisfactory, because the color of both is then too faint for accurate matching.

When the Hollander method was modified by mixing the very dilute gastric samples with an equal volume of the original test meal, the agreement between the two methods was improved in 8 out of 12 samples. The importance of correcting for excessive dilution is clearly shown by the fact that when no correction was made in the Hollander method, 76 per cent of the samples agreed within  $\pm 4$  per cent by the two methods, but when the correction was made in the Hollander method, 88 per cent of the samples agreed within  $\pm 4$  per cent.

When the dog with a gastroduodenostomy was studied, it was found that the Hollander method often failed to completely remove the bile so that the alkalinized gastric samples had a definite yellow tinge when compared with the standard test meal sample. In order to have made a satisfactory colorimetric reading, it would have been necessary to apply the picric acid correction to the standard solution. During the course of these studies it was again demonstrated that the picric acid correction gives highly satisfactory results.

#### SUMMARY

1 A comparison of the Hollander and Wilhelmj methods for determining the per cent of phenol red in gastric contents shows that down to approximately 50 per cent of phenol red in the gastric samples the agreement between the two methods is usually satisfactory. Below approximately 50 per cent discrepancies are frequent and marked. When the Hollander method is modified by correcting for excessive dilution of the gastric samples, the agreement is greatly improved.

2 Both methods may at times fail to entirely remove the bile from gastric samples containing excessive amounts, and it may be necessary to apply the picric acid correction.

3 When the Hollander method is modified to correct for excessive dilution of the samples the two methods are about equally reliable.

## REFERENCES

1. Wilhelmj, C. M., O'Brien, F. T., and Hill, F. C.: An Improved Gastric Test Meal, *Am. J. Digest. Dis. & Nutrition* 3: 319, 1936.
2. Wilhelmj, C. M., O'Brien, F. T., and Hill, F. C.: An Improved Gastric Test Meal and a Study of the Secretory Curve in Whole Stomach Pouches and in the Normal Intact Stomach, *Am. J. Physiol.* 115: 5, 1936.
3. Wilhelmj, C. M.: Test Meals and Methods of Gastric Analysis, *Am. J. Digest. Dis. & Nutrition* 4: 602, 1937.
4. Hollander, F., Penner, A., and Saltzman, M.: Determination of Phenol Red in Gastric Contents, *Proc. Soc. Exper. Biol. & Med.* 36: 568, 1937.
5. Folin, O.: *Laboratory Manual of Biological Chemistry*, New York, 1925, D. Appleton-Century Co., p. 125.
6. Peters, J. P., and Van Slyke, D. D.: *Quantitative Chemical Methods*, Baltimore, 1932, Williams & Wilkins, Vol. II, p. 38.
7. Mattice, M. R.: *Chemical Procedures for Clinical Laboratories*, Philadelphia, 1936, Lea & Febiger, p. 411.
8. Koch, F. C.: *Practical Methods in Biochemistry*, Baltimore, 1934, William Wood & Co., p. 268.

### A NOTE ON SOME PRESUMPTIVE TESTS FOR BENCE-JONES PROTEIN\*

G. M. DECHERD, JR., M.D., AND K. L. DICKENS, M.D., NEW ORLEANS, LA.

THERE is need for a procedure that will detect Bence-Jones protein during the routine examination of urine, or lacking this, one which will serve as a presumptive test for this substance and indicate further confirmatory tests. Bence-Jones proteinuria is rare, and routine detailed examination for it would be too time-consuming, but when it does occur, it is highly desirable to detect its presence. For it to manifest its characteristic precipitation at about 60° C., re-solution at boiling and reappearance when cool, the pH and salt concentration must have been suitably adjusted.<sup>1</sup> The inadequacy of the usual tests for proteinuria for demonstration of Bence-Jones protein at all consistently has been admirably proved by Osgood and Haskins.<sup>2</sup> These authors recommend two procedures: (a) To 5 c.c. urine, add 1 c.c. 50 per cent acetic acid and 3 c.c. 30 per cent (saturated) sodium chloride. They believe a precipitation appearing at room temperature "strongly suggests the presence of Bence-Jones protein," though a precipitate may occur when the urine contains 38 mg. per cent globulin. Their impression is that such urines are uncommon. (b) To 1 c.c. urine, add 0.1 c.c. 20 per cent sulphosalicylic acid solution. All proteins precipitate, but Bence-Jones protein redissolves at the boiling point.

The first presumptive test has found a place in several tests.<sup>3-5</sup> When we employed it in several instances of unexplained proteinuria, we were forced to the conclusion that it would give a positive result with nearly any urine in which protein could be found. In an effort to substantiate this impression quantitatively, we have analyzed urines from a representative group of renal diseases.

\*From the Department of Medicine, Louisiana State University School of Medicine, New Orleans.

Received for publication, March 24, 1938.

The total protein was precipitated by 10 per cent trichloroacetic acid, globulin by half saturation with ammonium sulfate, the supernatant fluid serving for albumin estimation. The amount precipitated by salt and acetic acid in the above concentrations was also determined. All precipitates were well washed, the second washing uniformly showing no color with Fohn's phenol reagent, and the various protein fractions were determined colorimetrically by the method of Andersch and Gibson.<sup>6</sup>

TABLE I

NO	CLINICAL DIAGNOSIS	TOTAL PROTEIN MG %	ALBUMIN MG %	GLOBULIN MG %	PPT BY NaCl AND ACETIC ACID	DILUTION STILL PPT	GLOBULIN IN FINAL DILUTION MG %
1	Acute nephritis	102	37	65	101	1:20	3.2
2	Amiloidosis	371	260	111	706	1:50	2.2
3	Pre-eclamptic toxemia	348	320	28	274	1:30	0.03
4	Febrile albuminuria	118	86	32	86	1:15	2.1
5	Congestive failure	202	180	22	175	1:15	1.5
6	Hypertension	179	169	10	174	1:10	1.0
7	Congestive failure	121	115	6	112	1:10	0.6
8	Congestive failure	210	190	20	191	1:20	1.0
9	Pre-eclamptic toxemia	795	708	87	780	1:70	1.2
10	Chronic nephritis	150	120	30	122	1:32	0.94
11	Subacute nephritis	693	545	150	675	1:80	1.9

Our results are tabulated. Each urine was diluted serially, and the highest dilution which gave a precipitate with salt and acetic acid was recorded. We calculated the amount of globulin which was present in this dilution, dilutions containing from 1 to 3 mg per cent still reacted positively. As may be seen from the table, urine containing over 38 mg per cent globulin is by no means uncommon. The bulk of the precipitate in each instance was albumin.

Our experience with the second procedure was more satisfactory, and we believe it conforms more nearly to the requirements of a presumptive test for Bence Jones protein. The salt and acetic acid method is still valuable as a means of precipitating the protein for removal of other urinary constituents, precipitation with it, however, is not suggestive evidence for the presence of Bence Jones protein.

## REFERENCES

- Hopkins, F. G., and Savory, H. A Study of Bence-Jones Protein and of the Metabolism in Three Cases of Bence Jones Proteinuria, *J. Physiol.* 42: 189, 1911.
- Osgood, E. E., and Hashins, H. D. Tests for Protein in Urine, Especially Bence Jones, *J. Lab. & Clin. Med.* 16: 575, 1931.
- Hawk, P. B., and Bergheim, O. *Practical Physiological Chemistry*, Philadelphia, 1937, P. Blakiston's Son & Co.
- Berglund, H., and Medes, G. *The Kidney in Health and Disease*, Philadelphia, 1935, Lea & Febiger.
- Osgood, E. E. *Laboratory Diagnosis*, Philadelphia, 1935, P. Blakiston's Son & Co.
- Andersch, M., and Gibson, R. B. Colorimetric Determination of Plasma Proteins. *J. Lab. & Clin. Med.* 18: 816, 1933.

## COMPARATIVE STUDY OF THE LAUGHLEN TEST FOR SYPHILIS\*

R. O. MUETHER, M.D., AND J. E. GREUTER, M.D., ST. LOUIS, MO.

SINCE Wassermann<sup>1</sup> first applied the principles of complement fixation to the diagnosis of syphilis, attempts have been made to simplify the test without sacrificing sensitivity and accuracy. The tests suggested have for the most part been only partially satisfactory, and the search continues for a test which is simple and inexpensive and yet of sufficient accuracy to warrant its use routinely on all patients. The need for such a test becomes more apparent when it is realized that the ravages of syphilis seem to be increasing in spite of our increased knowledge of the disease. This situation can only be rectified by a more careful examination of all individuals, and the popularization of blood tests for the detection of the disease. Increased blood testing, on the other hand, depends largely upon the facilities available and the price of the test to the individual. All new tests for syphilis must, therefore, present the following features: (a) it must be easily done; (b) it must be accurate, and (c) it must be economical. Any test which does not satisfy these criteria need not be considered in our search for more formidable diagnostic weapons with which to fight the disease.

Recently, two new methods for the detection of syphilis have been offered—one is the Laughlen<sup>2</sup> test and the other is the Ide<sup>3</sup> test. Both of these are more easily performed than most approved serologic tests for syphilis, and both of them are more economical than other serologic tests. It becomes necessary then to determine the accuracy and sensitivity of these tests, and it is with this problem that the present communication deals. Only the Laughlen tests will be discussed at this time. The result of the Ide tests will be published later.

It is not a simple matter to treat these various methods in an exactly comparable manner. The tests with which the staff is familiar will always appear in a slightly better light than a newly acquired test. This is further evidenced by the fact that the originator of a method usually gets a higher percentage of positive results than other laboratories using the same method.

The method of obtaining patients also plays a role in the end results. Patients should not be selected for such a study as this, and the number tested should be large.

### PROCEDURE

It was decided to do a thousand cases by Kahn<sup>4</sup> and Kline<sup>5</sup> tests, these being the serologic tests done in the laboratory routinely. On the same serum, the Laughlen test was to be performed by an individual not of the regular personnel of the serology laboratory. As far as possible, all the tests were done by

\*From the Laboratory Division, Department of Internal Medicine, University Hospital, St. Louis University School of Medicine.

Received for publication, April 8, 1938.

one of us (J. E. G.). Two hundred tests were done as a preliminary chiefly to become accustomed to the test and to work out a routine technique which would give the necessary speed to allow the completion of 40 to 60 tests in a reasonable time. When these were finished, the thousand routine blood tests were run; that is, every blood which came to the serology laboratory for a Kahn and a Kline was also subjected to the Laughlin test. When the thousand tests were completed, they were checked against the Kahn and Kline results.

#### TECHNIQUE OF THE LAUGHLIN TEST

The test is extremely simple, but requires careful attention to details. The test may be done on ordinary microscope slides, two tests to the slide. The ordinary medicine droppers have been found very satisfactory for the measuring of both serum and antigen. The serum may be inactivated or not. In fact, the test may be done on whole blood, but interpretation is much more difficult if whole blood is used. The actual test is done by mixing one drop of serum and one drop of antigen on a clean slide and mixing the two by gentle rotation or jarring. The specimen should be examined every few minutes by indirect light for the presence of clumping which appears as tiny but definite red specks in the cloudy mixture. If clumping occurs in the first two minutes, the serum is strongly positive; the less strongly positive sera take longer to produce the characteristic reaction. Any clumping which occurs after ten minutes or as the specimen begins to dry must be ignored. A known positive serum and negative serum must always be run as a control. The specimen may be examined under the microscope, although after a little practice this is not necessary.

#### PREPARATION OF THE ANTIGEN

The details of this procedure have been given by Laughlin in the *Canadian Medical Association Journal*, and will only be briefly summarized here. The antigen used by us was supplied through the courtesy of the Lederle Laboratories.

The preparation of the antigen may be divided into four steps: (1) preparation, (2) modification, (3) dilution of the modified antigen, and (4) standardization of the antigen to a certain sensitivity.

The antigen is prepared as is the Kahn antigen and is cholesterolized by adding 6 mg. cholesterol to each cubic centimeter of antigen. It is then modified by the addition of secharrach R to saturation and the further addition of tincture of benzoin compound. The modified antigen is then diluted by the addition of 5 c.c. 1.5 per cent saline (at 50° C.) to each 0.5 c.c. of antigen. If the antigen is satisfactory at this stage, it will be opaque and the particles will not settle out in twenty-four hours. This, then, is the stable inactive antigen and, before it can be used, it must be activated by the addition of an electrolyte. The amount to be added must be determined by the use of positive serum, a sufficient amount of antigen being added to produce clumping in from one to two minutes, with a known strongly positive serum. When this is determined, a comparable amount of electrolyte (10 per cent solution sodium chloride) may be added to the stable antigen as needed.

The sensitized antigen will only be found reliable for about seven days, and the quantity prepared should not exceed that which will be used in this period of time.

## RESULTS

It is rather difficult to interpret the results of various tests for syphilis, since no test is infallible. Every test so far devised is capable of giving both false positives and false negatives. The fact that, not infrequently, an individual may be a syphilitic and present no physical findings further complicates the matter.

TABLE I

	LAUGHLIN	KAHN	KLINE
Positive	138	142	133
Negative	867	858	867

TABLE II

	LAUGHLIN	KAHN	KLINE
False negative	18	2	8
False positive	24	1	2

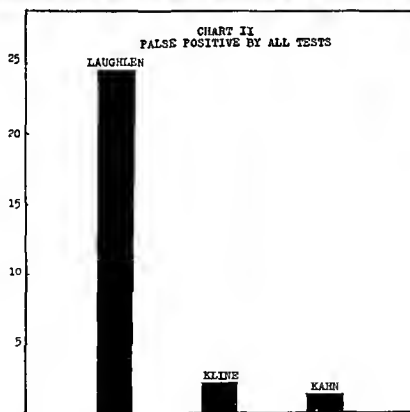
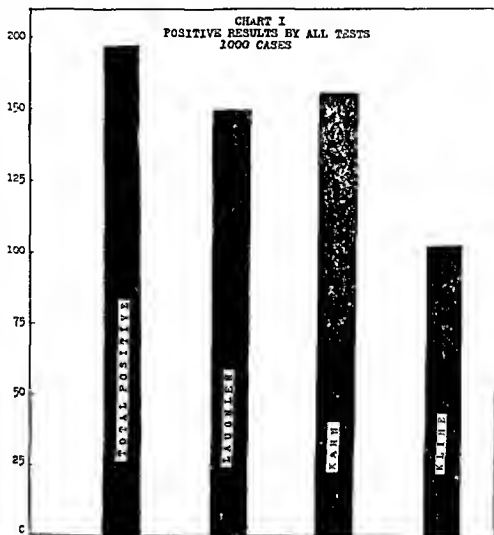
The Kahn test was the most accurate test in our hands, giving only one false positive reaction and two false negative reactions in 1,000 cases. The term, false positive, is here used to denote a positive serology by only one test in an individual who gives neither history nor physical findings consistent with diagnosis of syphilis. A false negative is, of course, the absence of positiveness by one test when other tests are positive and the patient is known to be a syphilitic. Chart I shows graphically the positive results. Table I gives the total number of

TABLE III

## ANALYSIS OF FALSE POSITIVES BY THE LAUGHLIN TEST

NUMBER	CLINICAL FINDINGS
12988	Gonorrhea, positive Neisserian fixation test; no findings suggestive of syphilis
12451	Chronic cervicitis (gonorrheal?); no findings suggestive of syphilis
12279	Gonorrhea; severe secondary anemia; positive Neisserian fixation test
5631	Pregnancy; pyelitis
12524	Cardiac; negative for syphilis
12918	First trimester of pregnancy
7222	Chronic cervicitis; negative for syphilis
13062	Cervicitis, cystocele, and rectocele; negative for syphilis
12229	Far-advanced tuberculosis
12743	Miliary tuberculosis
12423	Hyperthyroidism
12571	Fractured skull; no evidence of syphilis
12511	Appendicitis and cervicitis
12742	Sinusitis
12945	Parkinson's syndrome; no evidence of syphilis
12853	Arthritis; no evidence of syphilis
12936	First trimester of pregnancy; no evidence of syphilis
13025	Far-advanced tuberculosis
11156	Peptic ulcer
12715	Postoperative parotitis
12034	Gonorrheal salpingitis; no evidence of syphilis
H. H.	Blood donor; no evidence of syphilis
P. L.	Blood donor; no evidence of syphilis
M. W.	Blood donor; no evidence of syphilis

positive tests obtained by the various methods used and the total number of positives by each test. Table II gives the number of false positive and negative tests by the various methods. The Laughlen test was less efficient than either

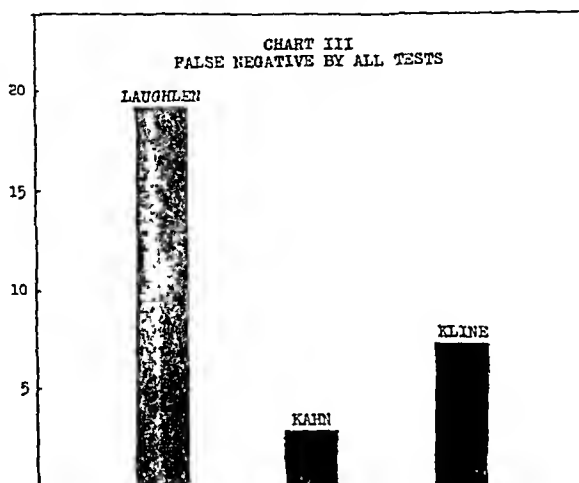


the Kahn or Kline. Table III gives an analysis of the false positive by the Laughlen technique, and Chart II gives the graphic representation of these. It will be noted that many of the false positives were obtained from individuals



with pelvic disease or pregnancy, while in several instances, the false positives were found in individuals suffering from advanced tuberculosis. Each patient who gave a false positive by the Laughlen test was re-examined clinically to rule out or discover a syphilitic history or lesion. In no instance did we succeed in doing this.

Table IV and Chart III present an analysis of the false negative obtained by the Laughlen technique. In two patients, the Kahn was the only test positive; but it will be noted that both of these patients had had a great deal of anti-syphilitic therapy, and when their histories were reviewed, it was found that



they had had positive Wassermann and Kline tests at the onset of their therapy. All the other cases, in which the Laughlen was falsely negative, had both Kahn and Kline tests positive. The Laughlen test was negative, when other tests were positive, in only one untreated case. We are, therefore, of the opinion that the Laughlen test is more easily reversed as a result of therapy than are the other

TABLE IV  
FALSE NEGATIVES BY THE LAUGHLEN TEST

NUMBER	KAHN	KLINE	REMARKS
12624	Positive	Positive	Chanere forty years ago; treatment at intervals
340	Positive	Negative	Under continuous treatment for two years
7588	Positive	Positive	Treatment for three years
327	Positive	Positive	Treatment for two years
785	Negative	Positive	Treatment for two years
12648	Positive	Negative	Treatment for one month
10690	Positive	Positive	History of syphilis for forty years, sporadic treatment
12266	Positive	Positive	Treatment for four years; history of chanere
266	Positive	Positive	Treatment for four years, serology variable from negative to positive
12425	Positive	Positive	Treatment for four years; had gumma
1950	Positive	Positive	Treatment for two years
196	Positive	Positive	Treated for two years; chanere six years before treatment
12139	Positive	Positive	Treated for two years; Neisserian fixation also positive
12233	Positive	Positive	Congenital, treated for four years
1553	Positive	Positive	Variable serology; has been under treatment for four years
12602	Positive	Positive	Under treatment for a year
12462	Positive	Positive	No history of chanere; no therapy
P. V. G	Positive	Positive	Under treatment two years; tertiary syphilis

tests This question is now being studied more carefully in a series of patients under treatment at the present time, and our conclusions will be presented when this work is finished

#### DISCUSSION

It should be pointed out that as our experience increased the accuracy of the test increased If we break up our series of a thousand cases into two groups, we find that 19, or about 80 per cent, of the false positive tests fall in the first 500 cases tested, while 8, or about 55.5 per cent of false negatives occurred in the first 500 cases These analyses of the cases as two groups, strengthen our belief that the Laughlin test is quite accurate in untreated cases, but is more readily reversed by therapy than are our other tests

The Laughlin test is a clean, quick and economical test, with a fairly definite end point The ability to determine the strength of the reaction on the basis of a time factor rather than on a quantitative basis is a distinct advantage, as there seems to be little reason to report any serologic test for syphilis by a series of plus signs The test for syphilis is far better reported as negative, positive, or doubtful

The Laughlin test, just as any other serologic test, requires a certain skill and training, and does not lend itself to use by everyone The large number of tests, which is necessary to produce efficiency in even a trained worker, seems to be sufficient reason for not recommending it as a test to be casually used in general practice It might well be used, we think, by the large laboratories because of its speed and economy when suitable controls can and will be done frequently The Laughlin test seems to be slightly less accurate than the Kahn test and compares fairly well with the Kline test

#### CONCLUSIONS

1 The Laughlin test is a simple, inexpensive, serologic test for the diagnosis of syphilis, with a sharp end point

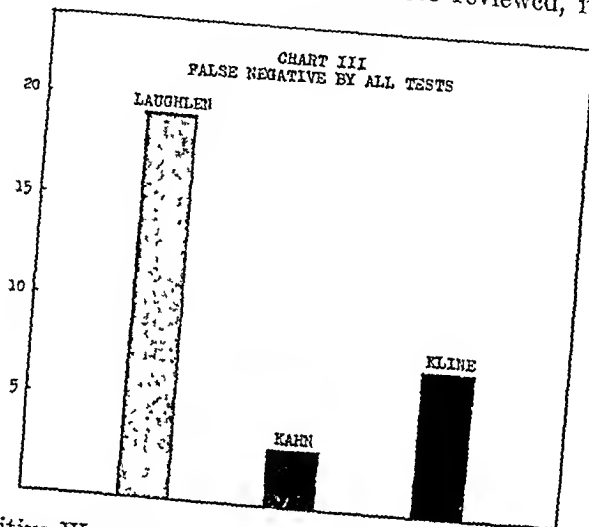
2 The Laughlin test is slightly less accurate than the Kahn test and compares fairly well with the Kline test

#### REFERENCES

- 1 Wassermann, A, Neisser, A, and Bruck, C Eine Serodiagnostische Reaktion bei Syphilis, *Deutsche med Wchnschr* 32 745 1906
- 2 Laughlin, G F A Rapid Test for Syphilis *Canad M A J* 33 179, 1935
- 3 Ide, Sobei, and Ide, Tamao Ide Test—New Color Test for Syphilis, *J LAB & CLIN MED* 21 1190, 1936
- 4 Kahn, R L The Kahn Test, Baltimore, 1928, Williams & Wilkins
- 5 Kline, B Microscopic Slide Precipitation Test for the Diagnosis of Syphilis, Baltimore, 1932, Williams & Wilkins

with pelvic disease or pregnancy, while in several instances, the false positives were found in individuals suffering from advanced tuberculosis. Each patient who gave a false positive by the Laughlen test was re-examined clinically to rule out or discover a syphilitic history or lesion. In no instance did we succeed in doing this.

Table IV and Chart III present an analysis of the false negative obtained by the Laughlen technique. In two patients, the Kahn was the only test positive; but it will be noted that both of these patients had had a great deal of anti-syphilitic therapy, and when their histories were reviewed, it was found that



they had had positive Wassermann and Kline tests at the onset of their therapy. All the other cases, in which the Laughlen was falsely negative, had both Kahn and Kline tests positive. The Laughlen test was negative, when other tests were positive, in only one untreated case. We are, therefore, of the opinion that the Laughlen test is more easily reversed as a result of therapy than are the other

TABLE IV  
FALSE NEGATIVES BY THE LAUGHLEN TEST

NUMBFR	KAHN	KLINE	REMARKS
12624	Positive	Positive	Chancre forty years ago; treatment at intervals
340	Positive	Negative	Under continuous treatment for two years
7588	Positive	Positive	Treatment for three years
327	Positive	Positive	Treatment for two years
785	Negative	Positive	Treatment for two years
12648	Positive	Negative	Treatment for two years
10690	Positive	Positive	Treatment for one month
12266	Positive	Positive	History of syphilis for forty years, sporadic treatment
266	Positive	Positive	Treatment for four years; history of chancre
12425	Positive	Positive	positive
1950	Positive	Positive	Treatment for four years; had gumma
196	Positive	Positive	Treatment for two years
12139	Positive	Positive	Treated for two years; chancre six years before treatment
12233	Positive	Positive	Treated for two years; Neisserian fixation also positive
1553	Positive	Positive	Congenital, treated for four years
12602	Positive	Positive	Variable serology; has been under treatment for four years
12462	Positive	Positive	Under treatment for a year
P. V. G	Positive	Positive	No history of chancre; no therapy
			Under treatment two years; tertiary syphilis

agglutination sometimes occurs with or without "quellung" in pooled sera, and indicates probable presence of at least two individual type reactions. We have been unable to obtain type sera above 32 for diagnostic procedure.

Sera of the Board of Health are used as controls. Five pools, however, are used, since our experience has shown that the complete pool of 1 to 32 with these sera is not as efficient as the smaller pools. The pools are made up as follows: (a) 5, 7, 8, 14, 18; (b) 10, 11, 12, 13, 15, 17; (c) 4, 6, 9, 16, 19; (d) 20, 21, 22, 23, 24, 25; and (e) 27, 28, 29, 31, 32. The Lederle pool of 1 to 32 was used to cross check the smaller pools and to facilitate finding the less frequent types.

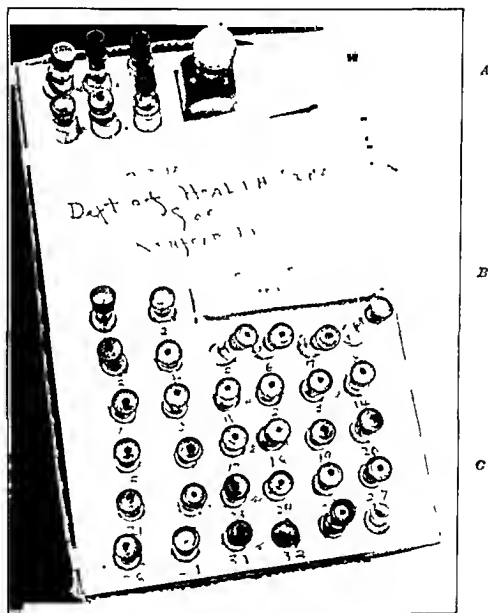


Fig. 2.—Work kit containing Department of Health sera controls. A, Pools, thin oil, pocket for cover slips; B, pocket for slides and loop, C, 32 individual sera

Since therapeutic sera prepared by the Board of Health and by Lederle are both used in the hospital, it was thought pertinent to scientific accuracy that both diagnostic sets be employed. We have kept two separate boxes of the diagnostic sera, slightly different in dimensions and size of bottle and carefully labeled, so that no confusion can result.

If any question of identification arises, cultures and specimens are referred to the Research Laboratory, Pneumonia Division, Board of Health. During the past two years only one serum, type 5, lost its potency, and only two strains of pneumococcus were found which did not correspond to any of the recognized 32 strains. Statistical findings will be reported in a separate paper.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**MENINGITIS**, Gonococcic, Branham, S. E., Mitchell, R. H., and Brainin, W. J. A. M. A. 110: 1804, 1938.

It seems likely that gonococcic meningitis is much more common than it is usually supposed to be. Hospital laboratories are usually much too busy to carry the identification of the causative microorganism to completion, and a gram-negative coccus of typical morphology which occurs intracellularly and extracellularly in the spinal fluid, and which is agglutinated by antimeningococcus serum, is usually assumed to be a *meningococcus*. Even when the study is carried further, definite criteria for the identification of the gonococcus are not well established and its identification is not easy. An absolute criterion is lacking; nevertheless, a careful study of cultures with consideration of all of their characteristics—type of growth, colony form, fermentation reactions, alkali solubility, and serologic reactions—will make an experienced worker feel quite sure of the identity of the strain in question.

Although gonococcic meningitis is usually secondary to gonococcic infection elsewhere, the case reported in this paper suggests that the gonococcus must be considered a potential cause of a primary meningitis without other gonococcic involvement.

**PORPHYRIA**, Studies in: Acute Idiopathic Porphyrin, Turner, W. J. Arch. Int. Med. 61: 762, 1938.

Acute idiopathic porphyria may be associated with excretion of uroporphyrin I in excess of uroporphyrin III.

Excretion of metal-porphyrin complexes appears to be characteristic of acute idiopathic porphyria.

Most of the color of the urine in acute idiopathic porphyria is usually due to the presence of pigments which have been called urofuscin.

Acute idiopathic porphyria is a familial disease, probably inherited as a dominant Mendelian characteristic.

The acute manifestations are alleviated by intravenous calcium therapy.

**MARROW**, Culture of Human, in Pneumococcic Infections, Osgood, E. E. Arch. Int. Med. 62: 182, 1938.

Culture of human marrow makes possible a type of control which is attainable in animal experimentation or in clinical investigation. In human marrow cultures, sulfanilamide exhibits a slight bacteriostatic action on pneumococcic infections which is increased by an increase in concentration. Even 0.3 unit per c.c. of specific antipneumococcus serum is more effective against the type I pneumococcus than is sulfanilamide alone. Sulfanilamide plus any given dose of antiserum, less than the amount which will by itself reduce colony counts to nearly zero, is more effective than corresponding doses of antiserum alone. These effects do not depend chiefly on phagocytosis. The results support the view that sulfanilamide renders the organism more vulnerable to bactericidal substances present in the serum. If the results of these in vitro experiments on the interaction of therapeutic and noxious agents in the presence of living human cells are applicable to infections in human beings, sulfanilamide therapy should be of value in pneumococcic pneumonia and might delay death in pneumococcic meningitis, but it will not prove as effective as even small amounts of type-specific antiserum. If used in conjunction with antiserum, it should further lower the mortality with the present doses of antiserum, or should give an equally low mortality with smaller doses of antiserum.

The use of both sulfanilamide and therapy designed to introduce or to develop specific bactericidins should be investigated further as a possible effective treatment for infections which are relatively resistant to the action of sulfanilamide alone.

**UROBILINOGEN** Excretion of Bile Pigment and Hepatic Function in Diseases of the Blood, Barker, W H Arch Path 62 222 1938

The excretion of urobilinogen has been studied in 74 cases of blood disease. The average daily output of urobilinogen in the feces is regarded as an index of the rate at which erythrocytes are being destroyed in the body.

Abnormally high values for urobilinogen in the feces were obtained in the cases of hemolytic anemia and in most of the cases of pernicious anemia in relapse, these values returned to normal after splenectomy in the cases of congenital hemolytic jaundice and after adequate liver therapy in the cases of pernicious anemia.

Based on these observations, the conclusion is reached that destruction of blood is increased in pernicious anemia, even though the disease may not be primarily a hemolytic anemia.

The anemia occurring in association with leukemia is occasionally hemolytic. In rare cases of aplastic anemia, a hemolytic factor is apparently active, splenectomy in 2 such cases was followed by temporary improvement.

Transfusion of well matched blood may at times be followed by a striking increase in the excretion of bile pigment, suggesting that the better part of the transfused blood is rapidly hemolyzed in such instances.

In 63 of the 74 cases of blood disease the hepatic function was studied by one or more of three tests, the test for urobilinogen in the urine, the bilirubin excretion test, and the hippuric acid synthesis test. Evidence of hepatic dysfunction was obtained in over 50 per cent of the cases, suggesting that a disturbance in the function of the liver may play a significant role in the pathogenesis of many of the blood dyscrasias.

**MENINGITIS, Tuberculous, Levinson Test in, Gleich, M Am Rev Tuberc 38 230, 1938**

Of 30 cases of tuberculous meningitis all gave a positive Levinson test. In 76.6 per cent of these patients tubercle bacilli were found in the spinal fluid. Twenty per cent of the cases showed tuberculosis at autopsy. In one case tuberculosis was noted at autopsy and tubercle bacilli were demonstrated in the spinal fluid.

None of 13 cases of nontuberculous meningitis had a positive Levinson test. One will occasionally find cases of nontuberculous meningitis or patients with other brain lesions who give a positive Levinson test. If it is remembered to refrain from performing a Levinson test on the spinal fluid which is contaminated by blood, or where serum has been previously given intrathecally, such pseudopositive Levinson tests will be avoided.

**GLUCOSE TOLERANCE TEST, the One Hour, Two Dose, Cooperstock M, and Galloway, J M Am J M Sc 55 1221, 1938**

The one hour, two dose dextrose tolerance test, previously found to be a specific, time saving, and convenient procedure for adults, was applied to infants and children.

The technique of the original test was modified to suit this age period. The micro method for determination of the blood sugar was found to be a reliable additional simplification of the procedure.

One hundred and two tests were performed on 82 subjects, consisting of 30 controls, 16 patients with diabetes mellitus of varying degree and duration, and 36 patients with non diabetic conditions.

The characteristics of the normal response to the one hour, two dose dextrose tolerance in a control group of subjects were found to be as follows: (1) a normal blood sugar level during fasting (average, 91 mg), (2) a more or less steep rise at thirty minutes (10 to 63 mg), and (3) a fall below the thirty minute blood sugar level at the sixty minute determination in 76.5 per cent of the tests, and an elevation above the thirty minute level at sixty minutes in 23.5 per cent, the total rise in the blood sugar level in no instance exceeding the normal level during fasting by more than 80 mg.

Application of these diagnostic criteria to the group of patients with nondiabetic conditions did not yield any unaccountable deviations.

The one-hour, two-dose dextrose tolerance test performed for the group of diabetic patients revealed the following features: (1) a normal blood sugar level during fasting or one above the normal limits (120 mg.); (2) a more or less steep rise at thirty minutes; (3) a hyperglycemic level at sixty minutes, the total rise exceeding 80 mg., with an occasional exception in cases of early diabetes mellitus of the low threshold type, and (4) glycosuria in at least the sixty-minute specimen.

Several abnormal curves were demonstrated for patients with nondiabetic conditions associated with deviations of normal carbohydrate metabolism.

The employment of the one-hour, two-dose dextrose tolerance test for infants and children demonstrated the important features of specificity, timesaving, and convenience. All these considered, the test offers an improved method for estimating dextrose tolerance in subjects of this age period.

**ANEMIA, Hyperchromic, in Infants, Stephenson, R.** *Am. J. Dis. Child.* 55: 1141, 1938.

A study was made of 64 normal white American-born boys and girls, ranging from 3 months to 2 years in age, and living under the same conditions in an institution having good pediatric supervision.

There being no other known variable, half of the infants were each given 30 gr. of iron and ammonium citrates (about 340 mg. of metallic iron) daily; the control infants were each given 6 gr. of ferrous sulfate (about 78 mg. of metallic iron) daily. Therapy was continued for three months, the effects being checked by estimations of red cell count, hemoglobin, and red cell volume.

As indicated by increases in hemoglobin, the ferrous iron proved to be as effective as the ferric. Apparently 10 gm. of hemoglobin per 100 c.c. of blood is the optimal level to be expected from administration of these forms and doses of iron, continued for three months, for infants of this age period.

The two groups having reached much the same average hemoglobin level at the end of three months, one group was then withdrawn from iron therapy while the other group continued to receive supplemental iron for an additional three months. The trend of the hemoglobin level was downward in those infants whose medication was discontinued, whereas it was upward in those for whom medication was maintained.

It seems probable that the increases in hemoglobin were the result of generous supplements of iron alone rather than of the traces of copper which contaminated both iron compounds.

This study confirms the claim that seemingly normal babies, between the ages of 3 months and 2 years, have a borderline anemia, and demonstrates that their hemoglobin levels can be raised to about 14 gm. per 100 c.c. by treatment with adequate iron without added copper. Increases in the number and volume of red cells also result, though they are less marked. Improvements in the infant's color, activity, and resistance to infection are believed to result, though they are difficult to measure. In this investigation ferrous iron produced a maximum effect with much smaller doses than ferric, and it caused no gastrointestinal disturbance. Some supplemental iron appears to be needed, up to the age of 2 years at least, to maintain the hemoglobin at this optimal level; it is possible that less than 6 gr. of ferrous sulfate would be an adequate maintenance dose.

**VITAMIN C, Urinary Output of Normal and of Sick Children, Bumbalo, T. S.** *Am. J. Dis. Child.* 55: 1212, 1938.

A macrochemical modification, both easy and accurate, of the Harris and Ray method for the determination of ascorbic acid is presented.

The urinary excretion of ascorbic acid of a normal child on an average diet is found to range from 11.2 to 71.2 mg. per twenty-four hours, directly dependent on the vitamin C intake.

The results of the study suggest that there is some vitamin C deficiency in all forms of tuberculosis in children. It is suggested also that there is some vitamin C deficiency in all febrile conditions in children.

The method of determination used follows:

(Tillman's reagent: 0.05 per cent solution of 2,6-dichlorophenolindo phenol made acid by the addition of 2 drops of glacial acetic acid 0.05 cc equivalent to roughly the amount of 0.025 mg of ascorbic acid.)

From 2 to 15 cc of urine is brought to a final volume of 15 cc and then brought to a pH of 4.7 by the addition of 0.02 cc of glacial acetic acid, Congo red being used as an indicator. It is then titrated with Tillman's reagent to an end point at which a blue color persists for at least fifteen seconds. Tillman's reagent is prepared so that 20 mg of the dye is contained in 100 cc of the dye solution. It is then standardized against a standard solution of ascorbic acid. Since it has been determined that 1 mg of ascorbic acid is the equivalent of 2.116 mg of Tillman's reagent, result may be easily calculated in terms of the amount of ascorbic acid excreted in twenty-four hours.

#### STREPTOCOCCI, Hemolytic Simplified Method for Grouping by Precipitin Reaction

Brown, J. H. J. A. M. A. 111: 110, 1918

The culture is grown in 5 cc of infusion broth containing 1 per cent of dextrose for from eighteen to twenty-four hours at 37° C. Many strains grow in the form of a sediment at the bottom of the test tube, others need to be centrifuged. All but about 1 cc of the supernatant broth is pipetted off and discarded. Two drops of metacresol purple indicator (0.04 gm dissolved in 60 cc of 95 per cent alcohol and then diluted to 100 cc with distilled water) are added to the remaining sediment suspension. From a drop bottle 2 per cent hydrochloric acid (about 6 per cent concentrated hydrochloric acid) is added until the indicator turns slightly pink (about pH 3.0). The tube of sediment is heated in a boiling water bath with occasional shaking for fifteen minutes and then cooled in running cold tap water for ten minutes. From a drop bottle 2 per cent sodium hydroxide is added until the color of the indicator passes through yellow and just begins to darken (about pH 7.5) but should not be noticeably purple. The tube is then centrifuged for about fifteen minutes, and the clear supernatant fluid used for the precipitin test. It has not been necessary to dilute the antigen.

#### TECHNIQUE OF THE TEST

On the bottom surface of a nearly optically perfect Petri dish (a satisfactory brand of such dishes is known as "Plano") rule 12 mm squares by means of a wax or diamond pencil. On the abscissa indicate the sera to be used, e.g., A, B, and C. On the ordinate indicate the antigens. Both inside and outside surfaces of the bottom of the Petri dish must be very clean and free from lint, dust, and finger prints, but need not be sterile. Within the appropriate squares and on the inside surface of the bottom of the dish, place one small (2 mm) platinum loop of antigen and one loop of serum, mixing the serum with the antigen as added, so as to make rather flat hanging drops when the dish is inverted. A platinum loop is specified because some of the cheaper substitutes give off alkali. To avoid carbon particles in the drops, it is essential to burn off the loop thoroughly, preferably after dipping it into water to remove most of the serum each time before flaming. It may be necessary to centrifuge the sera occasionally to free them from any particles of native precipitate. One should be careful not to form a precipitate by introducing a hot loop into the serum or antigen. Into the lid of the Petri dish is placed a disk of moist, but not too wet, white filter paper. With the bottom uppermost the bottom is placed into the lid of the dish.

*Reading of Results*—The assembled Petri dish is placed bottom up on the stage of a microscope, and the drops are observed through a 16 mm objective. The optimum illumination for observing particles of precipitate is secured by closing the diaphragm of the condenser until a small (about 2 mm) spot of light appears on the moist filter paper beneath the drop. With very little experience there need be no question about the interpretation of results. One soon learns to distinguish particles of foreign matter from the specific precipitate. In



the sera which the author used the result was usually apparent within fifteen minutes, and in one hour at room temperature it was fully developed. The plates were refrigerated overnight as a matter of routine, but this did not prove of any advantage. Seldom have there been any cross reactions within one hour. Sometimes as the precipitate is forming, it assumes a diffuse ground-glass appearance, which may be caused to form clumps by carefully rocking the dish.

Using the technique described, the author has grouped 236 strains of hemolytic streptococci of groups A, B, and C. Of these strains, 76 had been grouped by other investigators by the Lancefield technique, and the author's grouping was entirely in agreement with theirs. A few strains of other serologic groups were also tested and gave no precipitate with serum A, B, or C.

**SPUTUM EXAMINATION, Comparison of Direct Smear, Flotation-Concentration and Culture in, Smith, C. R. Am. Rev. Tuberc. 38: 57, 1938.**

A single technique is described for the simultaneous examination of sputum by direct smear, flotation-concentration, and culture.

A comparison is made of the results of simultaneous direct smear, flotation, and culture in the examination of 304 sputum specimens from 146 persons.

Sixty-three persons were positive, 28.5 per cent by direct smear, 69.8 per cent by flotation, and 88.8 per cent by culture.

Ninety-six specimens were positive; 19.7 per cent by direct smear, 62.4 per cent by flotation; and 88.5 per cent by culture.

Culture and flotation are overlapping and supplementary. Each test is capable of demonstrating tubercle bacilli, not detectable by the other. The routine performance of the two tests by a single technique is worth while.

*Procedure.*—The sputum of three days is collected in an 8 ounce bottle. A "likely particle" is selected with a wide-mouth pipette, and smeared on one end of a glass slide. The remaining sputum is shaken five minutes in a shaking machine, with an equal amount of 0.5 per cent sodium hydroxide. Two cubic centimeters of this preparation are removed and mixed with an equal amount of 2.5 per cent oxalic acid in a centrifuge tube. After incubation at 37° C. for thirty minutes, the acid mixture is centrifuged, and the sediment planted on two tubes of Petraghani's medium.

The sputum-sodium hydroxide mixture remaining in the bottle is subjected to the flotation test. It is incubated at 55° C. for thirty minutes, diluted to 200 c.c., and shaken five minutes with 1 c.c. of xylol. The creamy layer that appears at the top, after standing, is skimmed off with a pipette and spotted on the other end of the glass slide, three to five layers deep.

Direct and flotation smears on the same slide are fixed, stained by heating with carbol-fuchsin, decolorized, and counterstained with 1 per cent picric acid.

All solutions and glassware used must be sterile.

**FUNGI, Pathogenic, Production of Surface Growth of, on Culture Mediums, Arch. Dermat. & Syph. 38: 32, 1938.**

Phosphate, lactate, and a combination of the two increase the surface growth of certain pathogenic fungi. With combination or combinations will act depends on the organism. The base medium consists of 0.25 per cent cysteine and 4 per cent dextrose.

Phosphate, lactate, and the two together produce striking gross morphologic changes in the colonies of certain pathogenic fungi.

It is suggested that substances which result in the change of organisms from a sub-surface growth to a surface growth may be of value in bringing them to the surface in cutaneous infections, so that they can be cultured and more easily destroyed by antiseptics.

# *The Journal of Laboratory and Clinical Medicine*

VOL. 24

DECEMBER, 1938

No. 3

## CLINICAL AND EXPERIMENTAL

### INDICATIONS FOR CORAMIN IN CARDIOVASCULAR DISEASE

JOSEPH HENRY COWAN, M.D., JERSEY CITY, N. J.

IN AN earlier investigation,<sup>1</sup> several observations were made in the treatment of a varied group of cardiac conditions. When this study was conducted, particular attention was paid to the use of the same drug, coramin, in the treatment of a group of patients in the so called middle age period and decades subsequent, who showed varying degrees of impaired myocardial efficiency explainable on an arteriosclerotic and atherosclerotic basis.

There remains to be written a new chapter in medicine, dealing with this clinical entity. This type of degenerative myocardial disease has been described as (1) the failing heart of middle age, (2) chronic coronary artery disease, and (3) under a syndrome as multiple myocardial infarction. Correlated clinical and autopsy findings on these cases of hypertensive degenerative heart disease, giving histories of long periods of progressively impaired myocardial efficiency, bear out the pathologic patterns of numerous small myocardial infarctions resulting from multiple small closures with resulting diffuse replacement fibrosis.

Clinically, the patient referred to, is a sthenic individual, more frequently a male, in the beginning of the sixth decade, with an essentially negative past history, or a variable history of earlier hypertension, who has begun to notice breathlessness on exertion, discomfort over the sternum, varying from a feeling of uneasiness and pressure, to a stenocardia, occasional attacks of so called indigestion or acute indigestion, fullness and belching after meals or on exertion, and lacking the potentialities and dynamics of a year or six months earlier. This individual may continue along under a reduced program of activity, which he

the sera which the author used the result was usually apparent within fifteen minutes, and in one hour at room temperature it was fully developed. The plates were refrigerated overnight as a matter of routine, but this did not prove of any advantage. Seldom have there been any cross reactions within one hour. Sometimes as the precipitate is forming, it assumes a diffuse ground-glass appearance, which may be caused to form clumps by carefully rocking the dish.

Using the technique described, the author has grouped 236 strains of hemolytic streptococci of groups A, B, and C. Of these strains, 76 had been grouped by other investigators by the Lancefield technique, and the author's grouping was entirely in agreement with theirs. A few strains of other serologic groups were also tested and gave no precipitate with serum A, B, or C.

#### SPUTUM EXAMINATION, Comparison of Direct Smear, Flotation-Concentration and Culture in, Smith, C. R. *Am. Rev. Tuberc.* 38: 57, 1938.

A single technique is described for the simultaneous examination of sputum by direct smear, flotation-concentration, and culture.

A comparison is made of the results of simultaneous direct smear, flotation, and culture in the examination of 304 sputum specimens from 146 persons.

Sixty-three persons were positive, 28.5 per cent by direct smear, 69.8 per cent by flotation, and 88.8 per cent by culture.

Ninety-six specimens were positive; 19.7 per cent by direct smear, 62.4 per cent by flotation; and 88.5 per cent by culture.

Culture and flotation are overlapping and supplementary. Each test is capable of demonstrating tubercle bacilli, not detectable by the other. The routine performance of the two tests by a single technique is worth while.

*Procedure.*—The sputum of three days is collected in an 8 ounce bottle. A "likely particle" is selected with a wide-mouth pipette, and smeared on one end of a glass slide. The remaining sputum is shaken five minutes in a shaking machine, with an equal amount of 0.5 per cent sodium hydroxide. Two cubic centimeters of this preparation are removed and mixed with an equal amount of 2.5 per cent oxalic acid in a centrifuge tube. After incubation at 37° C. for thirty minutes, the acid mixture is centrifuged, and the sediment planted on two tubes of Petragnani's medium.

The sputum-sodium hydroxide mixture remaining in the bottle is subjected to the flotation test. It is incubated at 55° C. for thirty minutes, diluted to 200 c.c., and shaken five minutes with 1 c.c. of xylol. The creamy layer that appears at the top, after standing, is skimmed off with a pipette and spotted on the other end of the glass slide, three to five layers deep.

Direct and flotation smears on the same slide are fixed, stained by heating with carbol-fuchsin, decolorized, and counterstained with 1 per cent picric acid.

All solutions and glassware used must be sterile.

#### FUNGI, Pathogenic, Production of Surface Growth of, on Culture Mediums, Arch. Dermat. & Syph. 38: 32, 1938.

Phosphate, lactate, and a combination of the two increase the surface growth of certain pathogenic fungi. With combination or combinations will act depends on the organism. The base medium consists of 0.25 per cent cysteine and 4 per cent dextrose.

Phosphate, lactate, and the two together produce striking gross morphologic changes in the colonies of certain pathogenic fungi.

It is suggested that substances which result in the change of organisms from a sub-surface growth to a surface growth may be of value in bringing them to the surface in cutaneous infections, so that they can be cultured and more easily destroyed by antiseptics.

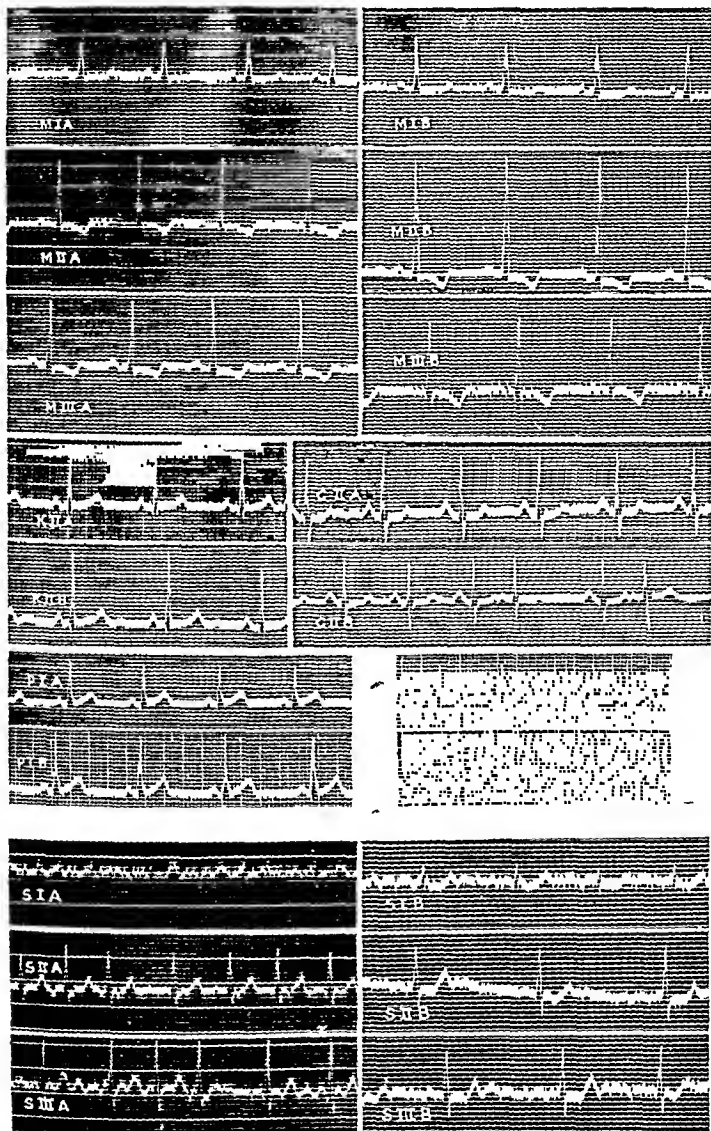


Fig 1—See bottom of opposite page for legend

action or by the ways discussed, we have to imagine how coramin is effective in conditions which are characterized clinically by the syndrome described earlier. Improvements of venous return flow, vital capacity, and ventilation are probably all factors in the improvement of coronary flow and myocardial efficiency.

The conclusions of the earlier observations are briefly:

1. The margin of safety for coramin is wide, as regards the single dose or use over prolonged periods.

2. Electrocardiographically, the drug produces an increase in voltage for all the main ventricular complexes, an increase that is generally well sustained. There occurs at times, lesser and more variable changes in other components, such as the T-wave and the P-R interval. A few observations suggest that the A-V conductivity may be slightly depressed in patients with badly damaged myocardia.

3. Subjective and objective changes which pointed to increased myocardial efficiency after sufficient dosage with coramin. This improvement may be due to (a) an action on the extrinsic cardiac nerves, or (b) a direct action on the heart muscle, or (c) an increase in coronary flow.

Some graphs illustrating the electrocardiographic changes described are given in Fig. 1. They are selected from the material in my first investigation and from this paper. In every case all three leads were taken, although in order to save space only the one that shows the most characteristic but common change in these cases after the use of coramin has been reproduced in some instances.

Seventeen cases of chronic coronary artery disease were studied in this later observation. This number comprised a group of 13 white males and 4 white females. The ages ranged from 47 years to 79 years. The entire group was urban dwelling in character. Three of the women were married housewives; one was an unmarried successful business woman. Of the males, two were physicians, eight were business men, two were newspapermen, and one was a laborer. All of these cases showed evidence, clinically, functionally, and physiologically, of impaired myocardial efficiency. Dyspnea was a cardinal symptom, varying from breathlessness on considerable exertion to breathlessness and cyanosis at rest.

The following case histories are characteristic of those studied.

CASE 1.—E. V. L., male, aged 63 years, first noted breathlessness in December, 1936, on climbing steps. His attention was particularly attracted to this symptom because of the relatively long recovery period which brought him to his physician for examination. His past history was essentially negative, except that he was a voracious eater and inclined to worry. He had high blood pressure and was overweight. He was placed on a low calorie diet, given some digitalis, and told to ease up his activities. In January, 1937, he had several attacks of "asthma" during his sleep which necessitated his having an injection for relief. His examination on February 15, 1937, revealed: blood pressure 168/100, heart rate 96, regular and rhythmical, heart enlarged to the left and downward, sounds of distant character and embryocardic in nature, liver enlarged several centimeters below the costal margin, neck vessels distended and pulsating, pitting edema of the feet and ankles extending up to the middle of the tibiae.

Fluoroscopy revealed moderate enlargement of the inflow and outflow tracts of the left ventricle, elongation and tortuosity of the aortic arch, with displacement of the barium filled esophagus to the right. I K G showed left axis deviation and high voltage. Bed rest for three weeks, restriction of fluids and diuretic established water balance, reducing the liver, abolishing the edema and the nocturnal dyspnea. On March 2 1937, coramin 30 minims twice daily, was commenced. On March 7, the patient no longer bedridden resumed moderate activity which he increased gradually. Since June he has carried on his business comfortably, even to the point of being able to climb steps without distressing breathlessness.

CASE 2—J J M, male aged 70 years, worked as a newspaperman until two years ago. At that time he suffered an acute coronary occlusion from which he recovered sufficiently to become ambulatory, but with such limited myocardial efficiency as to become dyspneic on the slightest effort. There had been no history of hypertension in earlier life. Physical examination showed a concentrically enlarged heart, diastolic pressure ranging above 100, and a diastolic murmur over the aortic area, probably due to a relative atherosclerotic aortic insufficiency. Fluoroscopy revealed a small amount of fluid in the right costophrenic sinus, both lungs were not well aerated and showed evidence of congestive failure, elongated, tortuous, and dilated aortic arch and thoracic aorta markedly enlarged inflow and outflow tracts of both ventricles. The neck vessels were distended and pulsating and the venous pressure was high. EKG showed low voltage and inverted T wave. All treatment had proved ineffectual in restoring compensation, there was no longer a response to diuretics. Coramin given in 30 minim doses twice daily was commenced on March 18 1937 and continued until May 27, 1937, with no improvement. Decompensation was growing more complete.

In summary it is obvious that there remained not a particle of reserve in Case 2. Myocardial fibrosis was probably so extensive, complete, and collateral, and coronary circulation so inadequate as to produce little or no response from any vasodilatory agent. In addition central depression of circulation will mitigate materially against aiding peripheral circulation. Those cases failing to show any change under the influence of coramin medication fall under this category.

#### CONCLUSION

Out of a group of 17 patients suffering from the chronic coronary artery syndrome, observed clinically over a period of several months, and treated solely with coramin medication, 12 were considerably improved, kept free from symptoms, and maintained in reasonably complete economic restitution, 3 patients were slightly improved, and 2 unchanged. As an addition to the armamentarium of cardiac therapy, coramin is suggested in therapeutic doses of from 20 to 30 minims twice daily, orally administered in fruit juices in the type of patient described earlier in this paper.

#### REFERENCES

- 1 Cowan, J H. Observations on Coramine. *Am J Med Sc* 193 673, 1937.
- 2 Stotland, O O, and Ginsberg, A M. Studies on Coronary Circulation, *J Pharmacol & Exper Therap* 60 396, 1937.

# DETERMINATION AND QUANTITATIVE ESTIMATION OF THE DECOMPOSITION OF CHLOROPHYLL IN THE HUMAN BODY\*

JOACHIM THEODOR BRUGSCH,† M.D., AND CHARLES SHEARD, PH.D.,  
ROCHESTER, MINN.

VERY little clinical information is available regarding the decomposition of chlorophyll in human metabolism, although much is known concerning the chemical structure of the chlorophylls per se and their relation to the blood pigments and porphyrins III, as has been shown by H. Fischer,<sup>1</sup> Conant,<sup>3</sup> and Willstätter.<sup>18</sup>

Chlorophylls differ from hemins in that they have magnesium instead of iron in the porphin nucleus and have different side chains, particularly the isocyclic ring. By reason of this more complicated structure, chlorophylls and their higher derivatives show typical spectroscopic absorption bands which are very different, in both position and number, from the simpler spectra of heminogenous porphyrins. There is also the possibility of rotation and alomerization. By proper chemical procedures chlorophylls in vitro may be changed to etioporphyrin III. Through these transformations, the phylogenetic relation, which may exist between hemin and chlorophyll in the natural development of pigments, can be made clearer. Therefore, theoretically, simple porphyrins might occur as final products in the human metabolism of chlorophyll.

Experimentally, however, it has not been possible to prove that increased coproporphyrinuria was produced in the human body after feeding green vegetables, such as spinach, as has been shown by Brugsch,<sup>2</sup> and by Franke and Fikentscher.<sup>9</sup> On the other hand, phylloerythrin<sup>13</sup> and, more recently, pyrroporphyrin (Rothemund<sup>15</sup>) have been obtained as products of the decomposition of chlorophyll in beef bile. The alteration of chlorophylls in the enteric tract of ruminants has been investigated by H. Fischer and his collaborators<sup>5, 6, 8</sup> and by Rothemund.<sup>14, 15</sup> The hydrochloric acid in the stomach seems to have a pronounced effect upon the chlorophyll molecule in removing magnesium and possibly also phytol and methyl alcohol. Rothemund has found pheophytin *a*, pheopurpurin 18, and phylloerythrin in the gastric mucosa of cows. Further changes in the intestines are brought about by extensive reduction, decarboxylation, loss of rotation, and other modifications which culminate finally in the formation of phylloerythrin, a substance which is a very important final product of the decomposition of chlorophyll in bovine metabolism.

\*From the Division of Biochemistry and Division of Physics and Biophysical Research, the Mayo Foundation, Rochester.

†Fellow in Biochemistry, the Mayo Foundation.

Received for publication, March 28, 1938.

There are a large number of steps in the decomposition between chlorophyll and phylocerythrin. Some of these compounds have been isolated and chemically analyzed by H. Fischer and his collaborators. The method of Willstätter is generally used to isolate the products of decomposition. This method is based on the fact that both porphyrins and products of the decomposition of chlorophyll are extracted from ether by specific concentrations of hydrochloric acid, depending on the basicity of the compounds. The concentration of hydrochloric acid necessary to extract them becomes proportionately lower as the change in decomposition of chlorophyll is increased. Willstätter defined the hydrochloric acid number as the percentage amount of this acid which will remove about two thirds of the dissolved substance from an equal amount of ether. Using pure preparations Willstätter obtained the data given in Table I.

TABLE I

THE RELATIONSHIP BETWEEN THE CONCENTRATIONS OF HYDROCHLORIC ACID AND THE EXTRACTION OF VARIOUS DECOMPOSITION PRODUCTS OF CHLOROPHYLL WITH ETHER

COMPOUND	HCL NUMBER	PERCENTAGE HCL ABLE TO EXTRACT PRACTICALLY TOTAL AMOUNT
Pheophytin <i>a</i>	29	32
Pheophytin <i>b</i>	35	—
Methylpheophorbide <i>a</i>	16	18
Methylpheophorbide <i>b</i>	21	23
Pheophorbide <i>a</i>	15	17
Pheophorbide <i>b</i>	19.5	22
Phytoclordin <i>e</i>	3	4.5
Phytorhodin <i>g</i>	9	11
Pyrroporphyrin	1.5	3
Rhodoporphyrin	3	4
Phylloporphyrin	0.75	1.5

## METHODS

1 *Extraction*.—We have employed Willstätter's principle to obtain the decomposition compounds of chlorophyll which may exist in human feces. Ten grams of the amount of feces, collected in a period of twenty-four hours and, if possible, after feeding a meat-free diet, were treated with acetic acid and transferred into ether. After the removal of porphyrins, compounds of chlorophyll could be withdrawn from the ether by successive extractions with 10 per cent hydrochloric acid, 25 per cent hydrochloric acid, 37 per cent hydrochloric acid, using ether and hydrochloric acid in the proportion of about 3:1 by volume. The extraction is performed as follows:

Ten grams of the collected feces are ground in a mortar, and about 10 to 15 cc of glacial acetic acid is added. To this mixture, 75 to 100 cc of ether is added, and the mixture is again ground, the ether extract is then transferred to an Erlenmeyer flask. The remainder of the feces is ground repeatedly by adding a few cubic centimeters of glacial acetic acid and ether until the resultant extraction of ether appears practically colorless. All extracts of ether, together with the remainder of the ground feces, are shaken together in a machine for two hours. Then the mixture is passed through an ordinary paper filter and washed in a separatory funnel with distilled water.



to remove the free acetic acid. The procedure is carried on until the wash water no longer turns blue litmus paper red.

The acid-free ether is shaken with small amounts of 5 per cent hydrochloric acid in order to remove the porphyrins. The shaking is repeated two to three times for fifteen minutes. The 5 per cent hydrochloric acid extract contains all the porphyrins but some protoporphyrin may remain in the ether, especially in cases in which the feces contain a large quantity of hemin compounds produced by the presence of blood or meat in the intestinal tract. This protoporphyrin may be obtained in the subsequent extraction with 10 per cent hydrochloric acid.

In the 5 per cent hydrochloric acid we have frequently found, by spectroscopic examination, derivatives of chlorophyll which are carried down along with the porphyrins. This is likely to occur in the presence of colloidal impurities, although the hydrochloric acid number of those compounds may be far higher than the concentration of hydrochloric acid used. In order to separate such chlorophyll compounds from porphyrins and to restore them to the ether extract, we have found it necessary to neutralize the 5 per cent hydrochloric acid extract with sodium acetate until Congo paper remains red. The porphyrins and chlorophyll compounds are then placed in a separatory funnel and are extracted by ether containing a few drops of glacial acetic acid. It is necessary to extract the neutralized solution of porphyrins and chlorophylls several times with ether, adding some glacial acetic acid to maintain an acid reaction in order to avoid the loss of chlorophyll derivatives. We have used the property of red fluorescence in ultraviolet light to determine the presence of small amounts of porphyrins or chlorophylls. Larger amounts of the compounds of the chlorophyll type may be determined with a pocket spectroscope, especially by observing the band in the red region. The ether-acetic acid extract must be washed with slightly alkaline ( $\text{Na}_2\text{CO}_3$ ) distilled water and later with pure distilled water. The process is repeated until the acetic acid is removed from the ether. Subsequently the porphyrins may be removed by repeated shaking with 5 per cent hydrochloric acid. The decomposition products of chlorophyll which have higher hydrochloric acid numbers remain in the ether. The derivatives of chlorophyll which are in solution in ether are added to the ether extract, previously obtained, which contains the largest part of the chlorophyll compounds.

The foregoing separation of porphyrins from chlorophyll derivatives obtained from human bile and feces is adequate because, from our observations, phylloerythrin does not appear in any appreciable amount as an end product of chlorophyll metabolism. It would be difficult to separate phylloerythrin from other porphyrins, because the hydrochloric acid number is about eight (Brugsch and Keys<sup>2</sup>).

After the removal of porphyrins, the various chlorophyll derivatives in ether are extracted, in turn, with 10 per cent, 25 per cent, and 37 per cent hydrochloric acid. Each extraction is done three times with the same concentration of hydrochloric acid, the relation of the volume of ether to the volume of hydrochloric acid being about 3 to 1. In this manner three hydrochloric acid fractions are obtained finally: (1) 10 per cent, (2) 25 per cent, and

(3) 37 per cent hydrochloric acid; the remainder is (4) the ether fraction. Each fraction is examined spectroscopically for the presence of absorption bands characteristic of chlorophyll derivatives.

2. *Quantitative Estimations of Chlorophylls and Their Derivatives.*—Various methods may be used for the quantitative estimation of the chlorophylls or their derivatives which may be present in the four fractions.

A. Colorimetric determinations: the comparison of the intensity of the green color with the intensity of a standard solution of chlorophyll. This method can be used only for pure preparations (Willstätter).

B. Fluorescence determinations: the comparison of the intensity of the red fluorescence, in ultraviolet light, of an unknown amount of chlorophyll

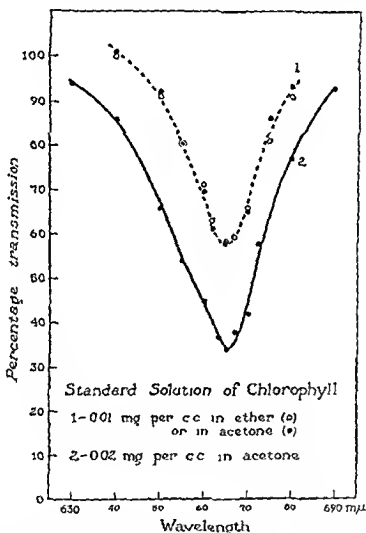


Fig. 1.—Curves of the spectrophotometric transmissions of solutions of crystallized preparations of chlorophyll in the region 630 to 690 millimicrons, 1, 0.01 mg. per c.c. of ether or acetone; 2, 0.02 mg. per c.c. of acetone

derivatives with a standard fluorescent solution. One of us (Brugsch<sup>1</sup>) has used the fluorescence of coproporphyrin as a standard to determine the relative amounts of compounds of chlorophyll decomposition extracted with 25 per cent hydrochloric acid from human feces, biles, and urines. The difficulty in using this method is the fact that solutions of chlorophylls and also solutions of their derivatives, which have high hydrochloric acid numbers, lose some or all intensity of fluorescence when they pass into a colloidal state. It is not possible, therefore, to keep standard solutions of chlorophyll for several days without loss of fluorescence. Hence the quantitative estimation of fluorescence as a measure of the amount of chlorophyll compounds which may be present is not satisfactory.

C. Spectrophotometric determinations; the measurement of the percentage of transmission (or absorption) of light in the red region of the spectrum by solutions of chlorophylls and similar compounds and the comparison with the transmission (or absorption) of light in the same spectral region by a standard solution of chlorophyll in the same condition (acetic acid ether). We have used a direct reading spectrophotometer, the so-called Keuffel and Esser color analyzer. This apparatus and methods for the spectrophotometric determination of porphyrin were described by Sheard, Osterberg, and Goeckerman.<sup>16</sup>

We have used standard solutions containing 1 or 2 mg. per 100 c.c. of crystallized preparations of chlorophylls. These materials were obtained from the American Chlorophyll Co., and were mixtures of chlorophyll *a* and *b*. For each spectrophotometric determination we have prepared a fresh solution of chlorophyll in ether in the presence of acetic acid. A standard solution, which contains the same amount of chlorophyll in acetone, gives the same spectrophotometric reading as does a solution in ether. Fig. 1 shows the spectrophotometric readings obtained in the region of the red absorption band of three standard solutions of chlorophyll; two of them (curve 1) contain 1 mg. of chlorophyll per 100 c.c. of ether or acetone, and the third contains 2 mg. per 100 c.c. of acetone. The maximal absorption occurs at approximately 665 millimicrons. For control purposes, we also made simultaneously qualitative spectroscopic examinations of the solutions and determined the presence of other absorption bands of chlorophyll derivatives. This procedure enabled us to rule out the presence of compounds which have similar absorption bands.

It should be emphasized that preparations of chlorophyll are affected readily by acid and other vapors in the room, as well as by light. Hence it is essential that a new supply of crystalline chlorophyll be used for the preparation of a standard solution. When solutions are prepared in this manner, the spectrophotometric readings at the point of maximal absorption (that is, 665 millimicrons) are practically the same (that is, 57 and 60 per cent, respectively). We have found that crystalline chlorophyll, which has been kept for a few months, may show a shift of the maximal absorption to (approximately) 670 millimicrons.

We have taken 58 per cent as the average value of the spectrophotometric transmission of the standard 1 mg. per 100 c.c. of a solution of chlorophyll at 665 millimicrons. We have neutralized the free hydrochloric acid in the hydrochloric acid extracts of chlorophyll derivatives from human feces with sodium acetate and sodium hydroxide and then transferred into ether with acetic acid before obtaining the spectrophotometric readings on solutions containing unknown amounts of chlorophyll derivatives. In every instance we made the spectrophotometric determinations as soon as possible after the transfer into ether. The amount of pigment can be determined from the laws of Bouguer and Beer,

$$\frac{C_1}{C_2} = \frac{-\log I_1}{-\log I_2}$$

in which  $C_1$  and  $C_2$  are the respective concentrations and  $I_1$  and  $I_2$  are the percentages of transmitted light at the maximal absorption value (that is, 660 to 670 millimicrons). The symbols  $C_1$  and  $I_1$  refer to the standard solution, in this case the wave length of maximal absorption is 665 millimicrons

QUANTITATIVE ESTIMATIONS OF DECOMPOSITION OF CHLOROPHYLL SUBSEQUENT TO THE INGESTION OF A GIVEN TYPE OF DIET

Table II gives the values of the concentrations (per 100 gm of feces) of the products of chlorophyll decomposition determined from the spectrophotometric readings and a substitution in the equation expressing the Bouguer-Beer law

TABLE II

AMOUNTS OF CHLOROPHYLL DERIVATIVES EXTRACTED BY VARIOUS CONCENTRATIONS OF HYDROCHLORIC ACID

CASE	10% HCl (MG PER 100 GM FECES)	25% HCl (MG PER 100 GM FECES)	7% HCl (MG PER 100 GM FECES)	ETHER (MG PER 100 GM FECES)
1	0.732	.925	15.2	11.0
2	.21	22.0	56.5	7.95
3	0.787	2.65	.24	Negative
4	0.689	2.45	10.16	1.05
5	0.612	17.725	40.71	7.525
6	0.341	7.72	23.8	0.576
7	Traces	3.55	1.395	3.355
8	Traces	4.12	1.8	0.55
9	Negative	Negative	2.40	Traces
10	Negative	Negative	7.40	0.858
11	Negative	Negative	10.792	Negative
12	Negative	0.172	4.08	1.5
13	Negative	0.527	1.095	0.86
14	Negative	Negative	.14	Negative
15	Negative	0.4575	41.31	?
16	Negative	.34	0.27	4.877
17	Negative	Negative	0.63	0.48
18	Negative	Negative	4.14	3.05
19	Negative	1.89	4.21	2.27
20	Negative	0.816	4.725	2.16

*1 Pheophytin Fraction (37 Per Cent Hydrochloric Acid)*—The results obtained show that the largest part of decomposed chlorophylls found in human feces cannot be extracted from ether with 10 per cent or 25 per cent hydrochloric acid, but only with 37 per cent hydrochloric acid. Therefore, according to our methods and data, the largest part of the chlorophyll decomposition in the bowels has not progressed beyond its initial stage. This fraction may be called the pheophytin fraction, the largest part being pheophytin. Curve 1 of Fig 2 shows the spectrophotometric transmission of this fraction after neutralization of the hydrochloric acid with sodium hydroxide and the transfer into ether.

It is well known that pheophytins in concentrated hydrochloric acid are very unstable. Pheophytin is converted into pheophorbide readily by the action of hydrochloric acid. After standing for a few hours, pheophytins in 37 per cent hydrochloric acid are changed completely to pheophorbides. Hence we have allowed the compounds of the pheophytin fraction to remain in the 37 per cent hydrochloric acid for several hours in order to convert them into

phorbides. The phorbides are transferred into ether and again extracted; initially with 18 per cent hydrochloric acid to obtain the pheophorbide *a*, and later with 22 per cent in order to obtain the pheophorbide *b*. The 18 per cent hydrochloric acid contains the largest part of the pigments. This fraction is taken into ether and esterified with diazo methane (Fischer and Riedmair<sup>7</sup>). After standing twenty-four hours, pheophorbide is converted into chlorintrimethylester. This compound can be crystallized from acetone methyl alcohol and recrystallized out of ether methyl alcohol. The typical prismatic crystals have a melting point of 210° C.

2. *Phorbide Fraction (25 per cent hydrochloric acid).*—The 25 per cent hydrochloric acid fraction of the decomposition of human chlorophyll often contains compounds which, according to their hydrochloric acid numbers, are phorbides. Investigations of H. Fischer and Stadler<sup>8</sup> revealed some products of the decomposition of chlorophyll in feces of ruminants, especially of sheep. They found compounds such as pheophorbide *a*, pyropheophorbide *a*, dihydro-

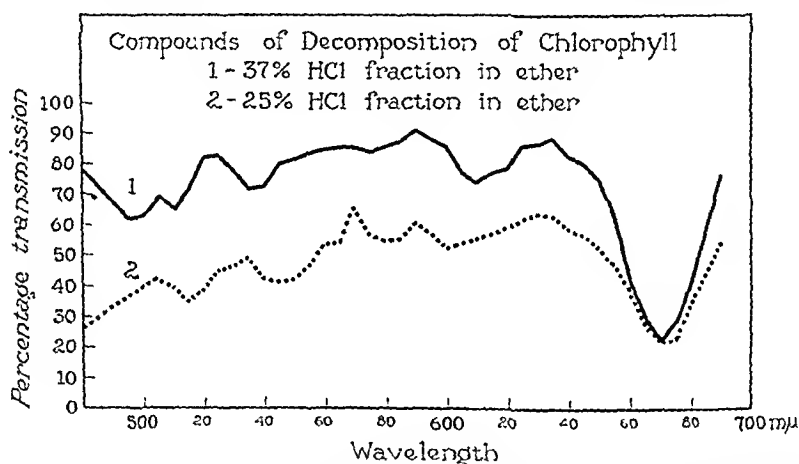


Fig. 2.—Curves of the spectral transmissions of the decomposition products of chlorophyll: 1, 37 per cent hydrochloric acid fraction in ether; 2, 25 per cent hydrochloric acid fraction in ether.

pheophorbide *a*, pyropheophorbide *b*, or so-called probophorbides. We have not been able to determine whether these are the same compounds which occur in human chlorophyll metabolism, and, if so, which of them occur. The curve of spectrophotometric transmission of such a mixture obtained by extraction from ether with 25 per cent hydrochloric acid is shown in Fig. 2, curve 2. The phorbide fraction may be fairly large in relation to the total excretion of chlorophyll products but not in excess of (approximately) 40 per cent, and comprises a variable part of the total amount of such pigments in the feces (see Table II).

3. *The 10 Per Cent Hydrochloric Acid Fraction.*—The amount of decomposition products of chlorophylls obtained from the feces is the smallest in the 10 per cent hydrochloric acid fraction. These decomposition products are detectable only after large amounts of chlorophyll have been ingested. We were not able to prove the presence of phylloerythrin in human feces or bile. If we take the spectrophotometric transmission of this fraction in 10 per cent

hydrochloric acid fraction (after the removal of impurities through repeated transfers and extractions between ether and 10 per cent hydrochloric acid), we obtain curves (Fig. 3, curves 1 and 2) which show three or four bands in both human bile and feces (obtained by treating 10 liters with acetic acid ether) instead of the two bands typical of phyloerythrin in 10 per cent hydrochloric acid.

The spectrophotometric curves obtained from 10 per cent hydrochloric acid extracts of bile and feces differed markedly from the spectral transmission curve of phyloerythrin in 10 per cent hydrochloric acid in that they exhibit the strongest absorption band in the red region (Fig. 3). These data and curves, and similar results, show that, in the decomposition of chlorophylls in the human body, compounds appear in bile and feces which are extractable from ether with 10 per cent hydrochloric acid and which may be differentiated readily from phyloerythrin in 10 per cent hydrochloric acid by the presence

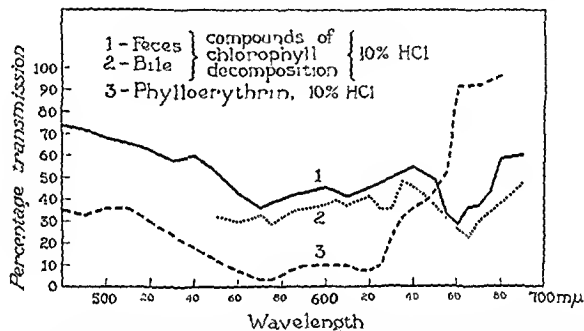


Fig. 3.—Curves of the spectral transmissions of the decomposition products of chlorophyll; 1, 10 per cent hydrochloric acid extract of feces; 2, 10 per cent hydrochloric acid extract of bile; curve 3 is the spectral transmission of phyloerythrin in 10 per cent hydrochloric acid.

of an absorption band in the red region of the spectrum. Furthermore, there is in ether a very noticeable difference spectroscopically, especially in the red region, between phyloerythrin and such compounds. The spectroscopic data, showing the presence and limits of the observed absorption bands, are: phyloerythrin in ether; I. 638 to 633 millimicrons, II. 597 to 579 millimicrons, III. 564 to 554 millimicrons, and IV. 528 to 513 millimicrons; in human decomposition of chlorophyll (10 per cent hydrochloric acid fraction); I. 673 to 656 millimicrons, II. 605 to 581 millimicrons, III. 565 to 554 millimicrons, and IV. 537 to 520 millimicrons.

4. *Chlorophyll Fraction (ether)*.—The chlorophyll remaining in the ether after all the extractions with different concentrations of hydrochloric acid may be, in part, unchanged. On the other hand, some chlorophyll may have been changed but cannot be extracted by hydrochloric acid in the presence of impurities, especially fats and colloids in the ether.

QUANTITATIVE ESTIMATION OF DECOMPOSITION OF CHLOROPHYLL  
AFTER INTAKE OF CRYSTALLINE CHLOROPHYLL

In order to exclude the possibility of changes in chlorophyll produced by the preparation of the food prior to its ingestion, we gave several hundred milligrams of standard chlorophyll in capsules to persons with normal metabolism and on a chlorophyll-free diet. The data of Fig. 4 show the increase in the excretion of the various decomposition products under the administration of chlorophylls given in doses of 100 mg. a day for four days. We have made the same fractionations with 10 per cent hydrochloric acid, 25 per cent hydrochloric acid, and 37 per cent hydrochloric acid, as were used in the investigations following the ingestion of a general type of meal. The pheophytin fraction is largely predominant. In one instance we were able to recover about 43 per cent of the 400 mg. of chlorophyll ingested. In another case

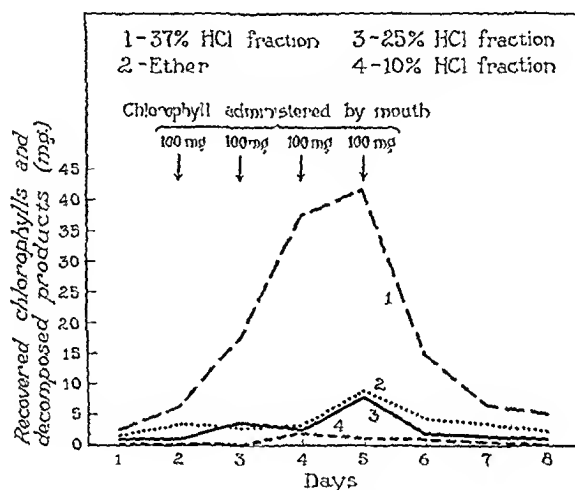


Fig. 4.—Curves showing the relationships between the intake of crystalline chlorophyll and the recovery of chlorophyll and its products of decomposition; 1, 37 per cent hydrochloric acid fraction; 2, ether fraction; 3, 25 per cent hydrochloric acid fraction, and 4, 10 per cent hydrochloric acid fraction.

there was excreted 72.7 per cent of chlorophyll subsequent to the first administration of 100 mg. of material.

The question arises as to whether such a loss of chlorophyll compounds is due to difficulties of the methods of extraction, the presence of impurities, absorption, and so forth, or whether, in addition to the type of chlorophyll decomposition we have investigated, there are other ways and means for the body to break down those pigments.

It has been suggested that the chlorophylls are decomposed to porphyrins in the course of metabolism and appear in the urine. We have investigated quantitatively this possibility, over a considerable period of time, in different individuals with and without the administration of chlorophyll. In the twenty-four-hour specimens of urine we have found coproporphyrin with 113.6 gamma as the highest value and, three days after stopping the feeding of chlorophyll, there was still 80.6 gamma in the urine. On the other hand, the amounts of

porphyrins in the feces during the intake of 400 mg of chlorophyll was 770 gamma as the highest value and only 486 gamma as its lowest value daily. It does not seem possible, therefore that the normal process of chlorophyll decomposition in the human body should lead to large amounts of porphyrins.

There is another possibility of the breakdown of such compounds. H. Fischer and Stadler have mentioned the appearance of reduced "leuco" compounds which could be isolated as phyloerythrin by reoxidation of the compounds extracted from the wash water. We have not investigated the presence and the importance of such compounds. The question also arises as to whether there is any direct relationship between the presence of larger amounts of huminoid pigments in urine and feces after the ingestion of food rich in chlorophyll. This problem in so far as we know has not yet received any serious investigation.

#### SUMMARY

1 Methods are described by which it is possible to extract and to estimate spectrophotometrically the amounts of decomposed products of chlorophyll which are found in human bile and feces.

2 The spectrophotometric determinations of various chlorophyll compounds in ether are based on the intensity of transmission in the red region (approximately 665 millimicrons) as compared with the transmission in the same region, obtained with a standard solution of crystalline chlorophyll *a* and *b*.

3 The products of decomposition of chlorophyll were separated into four fractions: (1) 10 per cent hydrochloric acid, (2) 25 per cent hydrochloric acid (phorbides), (3) 37 per cent hydrochloric acid (pheophytins), and (4) ether (chlorophylls and others).

4 The pheophytin fraction was found to be the largest portion of the products of chlorophyll decomposition recovered from human feces.

5 The presence of pheophytin *a* was proved by the conversion into pheophorbide *a* and crystallization into chlorotrimethyl ester (melting point, 210° C.).

6 The 25 per cent fraction was found to contain phorbides.

7 Compounds soluble in the 10 per cent hydrochloric acid fraction were obtained from human feces and human bile; spectroscopic analyses showed them to be different from phyloerythrin, both in the feces and in bile. We were not able to prove the presence of phyloerythrin.

8 It is possible to obtain the same fractions of chlorophyll products in the feces after the ingestion of pure crystalline chlorophyll with a chlorophyll free diet as under a general diet. The loss of the ingested crystalline chlorophyll may amount to as much as 57 per cent.

9 It is shown that the loss of chlorophyll is not due to the conversion into excreted porphyrins.

10 The possibilities concerning the fate of chlorophyll in human metabolism are discussed briefly.



## REFERENCES

1. Brugsch, J.: Untersuchungen des quantitativen Porphyrinstoffwechsels beim gesunden und kranken Menschen, IV Mit. Ztschr. f. d. ges. exper. Med. B. 98: 49, 1936; V Mit. Ztschr. f. d. ges. exper. Med. B. 98: 57, 1936.
2. Brugsch, J., and Keys, A.: Quantitative Separation and Estimation of Biological Porphyrins in Minute Amounts, J. Biol. Chem. In Press.
3. Conant, J. B., and Bailey, E. M.: Studies in the Chlorophyll Series IX. a. a. in the same journal. J. Am. Chem. Soc. 55: 795, 1933.
4. Fischer, H.: Chlorophyll, Chemical Rev. 20: 41, 1927.
5. Fischer, H., and Hendschel, A.: Über den biologischen Abbau der Chlorophylle. Ztschr. f. physiol. Chem. 198: 33, 1931.
6. Fischer, H., and Hendschel, A.: Über Phyllobombyrin and Probophorbide, Ztschr. f. physiol. Chem. 206: 255, 1932.
7. Fischer, H., and Riedmair, T.: Über die Aufspaltung von Chlorophyll "a" und seinen Derivaten durch Diazomethan, Liebigs Annalen 506: 107, 1933.
8. Fischer, H., and Stadler: Gewinnung von Dihydrophäophorbid "a" und Pyrophäophorbid "b" aus Schafkott, Ztschr. f. physiol. Chem. 239: 167, 1936.
9. Franke, K., and Fikentscher, R.: Die Bedeutung der quantitativen Porphyrinbestimmung mit der Lindermeier-Methode für die Prüfung der Leberfunktion und für Ernährungsfragen, Ztschr. f. physiol. Chem. 239: 171, 1935.
10. Godinho, A. P.: Über das Auftreten eines roten Farbstoffes nach Einnahme von reinem Chlorophyll, Biochem. Ztschr. 155: 90, 1925.
11. Hofstetter, Martha: Über den Nachweis von Chlorophyllabbauprodukten im Urin, Biochem. Ztschr. 155: 80, 1925.
12. Kitahara, Yoshitaka: Über die Ausscheidung von Chlorophyll derivaten aus dem Urin III, Biochem. Ztschr. 155: 97, 1925.
13. Marchlewski, L.: Über den Ursprung des Cholehämatis (Bilipurpurins), Ztschr. f. physiol. Chem. 45: 466, 1905.
14. Rothmund, P.: Occurrence of Decomposition Products of Chlorophyll. II, J. Am. Chem. Soc. 56: 2400, 1934.
15. Rothmund, P.: Occurrence of Decomposition Products of Chlorophyll. III. Isolation of Pyrroporphyrin From Beef Bile, J. Am. Chem. Soc. 57: 2179, 1935.
16. Sheard, C., Osterberg, A. E., and Goeckerman, W. H.: The Spectrophotometric Determination of Hematoporphyrin in Urine, J. LAB. & CLIN. MED. 15: 162, 1929.
17. Willstätter, R., and Stoll, A.: Untersuchungen über Chlorophyll, Verl., 1931, Julius Springer.
18. Willstätter, R.: Die Blattfarbstoffe, Handb. d. biol. Arbeitsmeth., Abt. I, Teil 11, p. 1, 1936.

## PRONTOSIL AND THE TREATMENT OF SPREADING PERITONITIS IN DOGS\*

JOHN O. BOWER, M.D., AND JOHN C. BURNS, M.D., PHILADELPHIA, PA., AND  
HAROLD A. MENGLE, M.D., FRANKLIN, N. C.

IT IS NOT without some hesitancy that we present this report of our investigation of the effect of prontosil upon spreading peritonitis of appendiceal origin, experimentally produced in dogs. The early reports of the efficacy of sulfanilamide against coccal infection, especially streptococcal, meningococcal, and gonococcal, aroused a wave of enthusiasm which resulted in regrettable untoward effects and fatalities. Now that reports and warnings of various ill effects have been published, perhaps the medical profession will view favorable conclusions from recent experimental work more deliberately than heretofore.

\*From the Department of Research Surgery, Temple University School of Medicine, Philadelphia, Pa.

Received for publication, March 28, 1935.

While our report deals with prontosil—disodium 4 sulfamido phenyl 2 azo 7 acetyl amino 1 hydroxynaphthalene 3 6 disulfonate\*—it is well known that this substance is closely related chemically to sulfanilamide and in the body is converted into and depends upon the action of that substance for its effects. For obvious reasons, therefore, we merely present the results we obtained in experimental animals without further comment. We have not used it for the treatment of spreading peritonitis in man in a sufficient number of cases to make a report.

We have already reported an extensive study of the bacteriology of appendiceal peritonitis in both man and dog.<sup>1</sup> In brief this study shows the predominating organisms in man to be *B. coli* (66 per cent of cases) and various strains of streptococcus (39 per cent of cases). In the dog, the incidence of *B. coli* was much lower (approximately 27 per cent) while that of the streptococcus remained about the same (32 per cent of cases). In both man and dog, *Cl. welchii* was present as the predominating anaerobic organism (60 per cent and 63 per cent, respectively). The high incidence of streptococci (of various strains) and *Cl. welchii* prompted this investigation.

The method of induction of spreading peritonitis in dogs and its treatment with horse serum, perfringens antitoxin and hypophyzed convalescent serum, have been previously reported.<sup>2</sup> The reasons for selecting dogs for this experimental work are of sufficient importance, however, to warrant repetition.

- 1 Dogs are always available for experimental work.

- 2 While the appendix of the dog is very much larger than that of the human being, especially when compared to the size of the intestine, it has a similar structure, and its base and mesentery can be easily ligated and gangrene readily induced.

- 3 The peritoneum of the dog is more resistant to infection than that of any other animal having an appendix. This is a decided advantage because the gradual development of the acute process makes possible accurate observation of gross changes and facilitates removal of tissue and exudate for histologic and bacteriologic examinations.

- 4 The constitutional reaction of the dog following an induced gangrene of the appendix is similar to that observed in the human being when the appendix is acutely inflamed. Temperature is moderately elevated, the heart rate is moderately increased, the dog vomits and remains in one position unless disturbed. Examination shows a neutrophilic leucocytosis, and the blood culture is invariably negative for microorganism. When rupture occurs, the temperature and pulse are greatly increased, the tongue becomes dry, the eyes lusterless, and the abdomen moderately distended.

- 5 In addition, the complications of mechanical ileus and metastatic abscesses do not develop frequently, so that the symptoms and signs accompanying these hazards do not have to be dealt with.

Following the induction of gangrene of the appendix, in approximately 50 per cent of the dogs a small localized abscess forms, and they recover without any treatment. The intraperitoneal changes which accompany recovery closely

\*The prontosil for this investigation was furnished by the Winthrop Chemical Co.

resemble those observed in the human being. A certain percentage, however, will develop peritonitis and die. This percentage is increased by the administration of laxatives, just as in the case of man. The time of administration, kind and quantity of laxative administered, influence the mortality decidedly.

#### TECHNIQUE

1. *Selection of Animals.*—Dogs were selected at random, except that the control and treated dogs were of approximate size and weight. Pregnant dogs and those weighing less than 5 kg. were not used. Occasionally dogs were available that had been operated upon previously; but those that had abdominal operations and had been returned to stock within thirty days were not used. No effort was made to prepare the dogs for operation, except that they were not fed that morning.

2. *Operative Technique.*—All the operations were carried out under ether anesthesia. The abdomen was shaved, scrubbed with ether, and painted with 3.5 per cent tincture of iodine. Using aseptic technique, a right rectus incision, about 10 cm. long, was made, the appendix located, drawn up, dissected free of its mesenteric attachment, and all bleeding vessels clamped and separately ligated with fine silk. A double ligature of silk was then tied tightly around the appendix as close to its base as possible. After inspection to insure complete hemostasis, the abdominal contents were replaced and the wound closed in layers, using silk throughout and re-enforcing the fascia with interrupted sutures. Drains were not used. During the operation, precautions were taken to avoid the escape of blood into the peritoneal cavity. No dressings were applied.

3. *Administration of Laxatives.*—Early in our study of appendiceal peritonitis we found that by giving 30 c.c. of castor oil to the average dog *twenty-four hours after operation*, a spreading peritonitis could be induced in about 70 per cent of the cases. The mortality of this particular series was 68.5 per cent; 91.9 per cent of the deaths were due to spreading peritonitis. We then gave 30 c.c. of castor oil to a group of animals *directly after operation*. The mortality rose to 78.3 per cent, and 94.5 per cent died of spreading peritonitis. An average dose of 54 c.c. of castor oil was then given to a third group of dogs *immediately after operation*; the mortality rose to 91.7 per cent, and 100 per cent died of spreading peritonitis.<sup>2</sup> These experiments proved that the time of administration of laxatives and the quantity of laxative administered influence the outcome. Every animal that received 2 ounces of castor oil the day of operation, or twenty-four hours following the operation, died of spreading peritonitis.

4. *Postoperative Care.*—The animals were placed in metabolic cages and allowed to recover before being returned to the animal house. Food and water were placed before them. They were disturbed only when brought to the operating room for the administration of prontosil. They were returned to the animal rooms immediately after this procedure, with as little handling as possible. Special care was taken to avoid squeezing or massaging their abdomens.

Post-mortem examinations were carried out in all instances as soon as possible. If post-mortems were not done to determine the cause of death, if an

animal died of pneumonia or other complications within ten days, or if at subsequent operation the appendix was found to be intact, showing that the ligature had slipped, then the record was discarded.

# RESULTS OF THE TREATMENT WITH PRONTOSIL OF INDUCED SPREADING PERITONITIS IN DOGS

Twelve dogs were used as a control group for comparison with the treatment group. The average weight of these control dogs was 11.6 kg. The appendix was ligated at its base, and the mesentery was stripped free and

TABLE I  
PRONTOSIL  
*Animals That Recovered*

DOG NO	WEIGHT kg	PRONTOSIL TOTAL DOSE cc	AVG DOSE cc IN 5 HR	NO LIVED	SUBSEQUENT OPERATION APPENDIX ABSORBED
96	11	55	11	1	1
111	9	45	9	1	1
570	15	75	15	1	1
760	10	50	10	1	1
763	6.6	32.5	6.5	1	1
765	10	50	10	1	1
777	11.5	51.75	8.6	1	1
784	15	65	13	1	1
786	18	90	18	1	1
788	14	70	14	1	1
791	15	60	15	1	1
	121	55.6	11.8	11	100 per cent

PRONTOSIL  
*Animals That Died*

DOG NO	WEIGHT kg	PRONTOSIL TOTAL DOSE cc	AVG DOSE cc IN 5 HR	NO DIED	AVG HR LIVED AFTER OP	PATHOLOGICAL SPREADING PERITONITIS
94	10	30	10	1	36	1
100	6	30	6	1	45	1
101	10	50	10	1	120	1
102	5	25	5	1	48	1
110	8	24	8	1	24	1
146	14	56	14	1	48	1
754	15	75	15	1	226	1
759	9	40.5	8.7	1	80	1
769	9 "	37.5	7.5	1	48	1
787	13	65	13	1	122	1
793	11	49.5	8.2	1	72	1
	10 "	43.9	9.4	11	80.18	100 per cent

ligated separately. An average dose of 54 cc of castor oil was given immediately after operation. The mortality was 91.7 per cent, 100 per cent died of spreading peritonitis, and the average number of hours those that died survived the operation was 66. In the treatment group were 22 dogs. The average weight was 11.03 kg, the average weight of those that lived was 12.1 kg, and of those that died, 10.03 kg. With two exceptions, the dose of castor oil was 60 cc. Two dogs in the treatment group received only 45 cc of castor oil,

instead of 60 c.c. One of these dogs lived and one died, No. 763 and No. 793, respectively. In the treatment group, those that recovered or died received the same amounts of castor oil.

*Dose and Method of Administration of Prontosil.*—Each dog was given 1 c.c. of prontosil per kilogram of body weight every eight hours, day and night. It was given intramuscularly, because our previous experience with perfringens antitoxin had shown that the intramuscular injection was more effective.<sup>2</sup> Each of the 22 dogs received an average dose of 51.2 c.c. total of prontosil. Those that recovered received an average total dose of 58.6 c.c., and those that died, 43.9 c.c. The average individual dose of those that lived was 11.8 c.c.; of those that died, 9.4 c.c. The diminished total dose of those that died is due to the fact that death interrupted further treatment. The animals that died survived the operation an average of 80.1 hours. The mortality of this group was 50 per cent: 11 dogs lived, 11 died. In those that lived, subsequent operation showed that the appendix was completely absorbed in every instance. Of those that died, 100 per cent died of spreading peritonitis. Table I shows the weight, total dose of prontosil, and the outcome of each individual dog.

#### SUMMARY

Twenty-two dogs, average weight 11.1 kg., were operated upon under ether anesthesia. Gangrene of the appendix was induced by ligating the blood vessels in the mesentery and the base of the appendix.

Spreading peritonitis was induced by the administration of 58.6 c.c. (average dose) of castor oil immediately after operation.

One cubic centimeter of prontosil per kilogram of body weight was injected intramuscularly every eight hours, day and night, until the animal had either died or shown sufficient improvement to indicate he would recover. The mortality of this group was 50 per cent.

A control group of 12 animals, average weight 11.6 kilograms, was operated upon as described, given 54 c.c. (average dose) of castor oil immediately after operation, but received no treatment. The mortality of this group was 91.7 per cent.

#### REFERENCES

1. Bower, John O., Burns, John C., and Mengle, H. A.: The Bacteriology of Spreading Peritonitis Complicating Acute Perforative Appendicitis, A Clinical and Experimental Study, *Surgery* 3: 645, 1938.
2. Bower, John O., Burns, John C., and Mengle, H. A.: Induced Spreading Peritonitis Complicating Acute Perforative Appendicitis, *Surg. Gynec. & Obst.* 66: 947, 1938.

## THE ACTION OF CHEMICAL AND PHYSICAL AGENTS ON *CLOSTRIDIUM WELCHII* AND ITS TOXIN\*

FRANCIS E. COLIEN, PH.D., OMAHA, NEB.

SINCE the early investigations of Minck (1896), considerable work has been reported on the effects of x-rays and ultraviolet light on microorganisms. Moore and Kersten (1936) prepared a nontoxic vaccine of *Shigella dysenteriae* (Shiga) by irradiation of saline suspensions of the organisms with soft x-rays. In 1937, by the same method, they inactivated encephalitis virus (St. Louis type). Blank and Kersten (1935) showed that a modified extract agar could be so altered by the action of soft x-rays that it would no longer support the growth of *Bacillus subtilis*.

This preliminary report deals with a study of the effect of x-rays on the toxin and organisms of *Clostridium welchii*. Kelly (1933) and Kelly and Dowell (1936) reported on the use of x-rays in the treatment of gas gangrene. In a later paper, Kelly and others (1937) were able to show some helpful effects by treating guinea pigs injected with *Clostridium welchii*, or its toxin, with x-rays. By irradiation of these animals some therapeutic action was shown. In some of the experiments, over 50 per cent of the animals treated lived while the controls died in a few days with typical gas gangrene. In other cases, the x-ray treatment seemed to increase the time, by several hours or days, between the inoculation and death of the animals. Irradiation also destroyed or decreased the gas in the tissues in a number of the animals.

### APPARATUS AND TECHNIQUE

Two cultures of *Clostridium welchii* (No. 88 and 88 a) isolated from fatal cases of gas gangrene were used in this study. The cultures were purified by the usual anaerobic methods.

The toxin was produced by growing the organisms in beef infusion broth, pH 7.8, containing 0.1 per cent dextrose and sterile guinea pig tissue. The cultures were incubated for twenty to twenty-four hours at 37° C, centrifuged and filtered through a Berkefeld N filter. Six-tenths of a cubic centimeter of this filtrate killed 350 gm pigeons in eight to ten hours.

Standard x-ray equipment and tubes were used. The tubes were operated at a voltage of 90 kv. and a current of 5 Ma. The material was irradiated for eight minutes at a distance of 30 cm. from the copper target. A 1 mg. aluminum filter and a cone were used throughout the work.

\*From the Department of Bacteriology and Preventive Medicine, Creighton University School of Medicine, Omaha.

Received for publication, April 6, 1938

## EXPERIMENTAL

In these experiments we were primarily interested in studying the effects of x-rays and biochemical substances on *Clostridium welchii* and its toxin. The material to be irradiated was placed in long slender glass tubes (1.5 by 20 cm.) and sealed with a rubber cap. The thickness and type of glass used in these tubes was taken into consideration in determining the radiation, which was equivalent to that used in treating patients with gas gangrene and similar infections.

In order to simulate conditions found in gas gangrene infections, the following substances were irradiated: 6 c.c. of a toxic filtrate with sterile guinea pig tissue, 6 c.c. of a toxic filtrate, 6 c.c. of an eighteen-hour culture of *Clostridium welchii* in beef infusion broth in the presence of sterile tissue, and a toxin leucocyte mixture consisting of 1.5 c.c. of a 0.5 per cent suspension of human cells in saline and 1.5 c.c. of toxin. The materials were prepared in duplicate so that one tube could be treated with x-rays while the other served as a control. The tubes containing the various substances were kept at room temperature for four hours before the first x-ray treatment. They were irradiated for a period of eight minutes every twenty-four hours for five successive days, and kept at room temperature throughout the experiment. Four hours after the first, second, and fifth treatments, the toxicity of the substances was tested on pigeons. The viability of the organisms was also tested by subculture in beef heart medium. The results of these tests are summarized in Table I.

TABLE I  
ACTION OF X-RAYS ON *Cl. welchii* AND ITS TOXIN

MATERIAL INJECTED INTO PIGEONS*	TIME OF DEATH AFTER FIRST TREATMENT	CONTROL	TIME OF DEATH AFTER SECOND TREATMENT	CONTROL	TIME OF DEATH AFTER FIFTH TREATMENT	CONTROL
Culture 0.6 c.c.	6.5	6	7	6	7	6.5
	8.5	7	10.5	8.5	8	7
Toxin and tissue 0.6 c.c.	20	18	22	17	Lived	Lived
					Lived	Lived
Toxin 0.6 c.c.	8	8	8.5	8	7.5	8
					8	9
Toxin and leucocytes 1.2 c.c.	9	8	9.5	9		
	14	9	14	13.5		

\*Weight of pigeons 350 to 400 gm.

Similar results were obtained when the above experiment was repeated using a more toxic filtrate given in 0.3 c.c. dosage.

No marked decrease in toxicity or viability was observed after irradiation of toxin, toxin-leucocyte suspension, or cultures of *Clostridium welchii*. Toxin in the presence of sterile tissue, treated or not treated with x-rays, gradually loses its toxicity for pigeons. This loss of toxicity suggests that tissue may absorb toxin or contains some definite substances which when released from tissue, either by means of x-rays or by disintegration, destroy toxin. In order to learn more about this action, an attempt was made to study the effect on toxin of various substances that are present in tissue and that might be released by x-rays.

A 3 per cent solution of hydrogen peroxide and 2 per cent solutions of cystine and glutathione were mixed with toxin allowed to stand at room temperature for six hours and injected intramuscularly into pigeons weighing 350 to 400 gm. In one experiment mixtures of 0.6 cc of toxin and 1 cc each of hydrogen peroxide, cystine and glutathione were injected. The pigeons that received the hydrogen peroxide and glutathione lived; those that received the cystine died in forty hours and the controls died in eight hours. In another experiment pigeons were injected with mixtures of 0.8 cc of toxin and 0.2 cc of hydrogen peroxide and 0.5 cc each of cystine and glutathione. The pigeons receiving the hydrogen peroxide lived; those receiving the cystine died in thirteen hours, the glutathione in forty hours and the controls in seven hours. Three pigeons were injected with each of the toxin-chemical mixtures used in the above experiments. The results seem to indicate that certain chemical substances may play some part in detoxifying or altering toxin.

An attempt was made to determine the suitability of rabbits in studying the effects of x-rays on gas gangrene. Mature animals weighing 3500 to 4000 gm, and young animals of 800 to 900 gm were used. No definite lesions of gas were produced in 4 mature rabbits injected intramuscularly with 0.5 cc of a twenty-four hour culture of *Clostridium welchii*. In a series of 8 young rabbits (four to five weeks old) a small perforating necrotic lesion appeared in five days and gradually increased in size until the entire muscles of the thigh were involved. The muscle tissue became soft with some evidence of suppuration, and was gradually digested until the femur was exposed. There was no evidence of gas at any time.

#### DISCUSSION

Many investigators have found injurious or lethal effects produced on bacteria by x-rays, while others have failed to detect such effects. The results observed may be due to differences in the experimental conditions or to the nature of the organisms used. Lacissague (1928) suggested that bacterial suspensions may show extreme degrees of variation in resistance to radiation due to some physiologic peculiarity. In our experiments we were not able to show that x-rays destroyed either the toxin or organisms, with an amount of irradiation equivalent to that used in treating infections in man. There is the possibility that we were attempting to destroy too great an amount of toxin in proportion to the dosage of x-rays used or that toxin is not destroyed by x-rays. Further experiments are being conducted using smaller amounts of toxin.

There are substances present in tissue such as catalase, glutathione and cystine (present through oxidation of glutathione), which may be liberated or stimulated to increased activity by x-rays. These substances may destroy or alter toxin, possibly through an oxidation process. The catalase present in tissue may be modified or destroyed by irradiation, and thereby permit hydrogen peroxide to accumulate with the result that the toxin is altered. Our evidence as yet is only fragmentary, but may help to explain the non-specific effects of x-rays in the treatment of bacterial infections.



Desjardins (1935) believed that the destruction of infiltrating leucocytes, with the subsequent liberation of antibodies and other protective substances, plays a part in the rapid action of x-rays in curing certain acute and chronic infections. Although our preliminary experiments are not conclusive, we were able to show that leucocytes had little or no effect on toxin in the absence of living tissue.

Cornell (1925) produced chronic infections in mature rabbits with *Clostridium welchii*. Rabbits injected subcutaneously showed no marked symptoms, local reactions, or the presence of gas. We obtained similar results in mature animals, but were able to produce extensive local lesions in young rabbits; no gas was detected. There is a possibility that young rabbits may be of value in studying acute *Clostridium welchii* infections.

#### SUMMARY

1. No marked decrease in toxicity or viability was observed after irradiating toxin or cultures of *Clostridium welchii* with soft x-rays.

2. Considerable amounts of toxin may be absorbed or altered in the presence of sterile tissue.

3. Certain substances, such as glutathione, hydrogen peroxide, and to a slight extent cystine, may play some part in detoxifying or altering toxin in tissue.

4. Extensive local lesions were produced in young rabbits with pure cultures of *Clostridium welchii*.

The author acknowledges his appreciation to Dr. J. F. Kelly and Dr. D. A. Dowell, of the Department of Radiology, for their assistance in x-raying the materials.

#### REFERENCES

1. Minck, F.: Zur Frage über die Einwirkung der Röntgen'schen Strahlen auf Bakterien und ihre eventuelle therapeutische Verwendbarkeit, München med. Wchnschr. 43: 100, 1896.
2. Moore, H. N., and Kersten, H.: A Preliminary Note on the Preparation of Non-Toxic Shiga Dysentery Vaccines by Irradiation With Soft X-rays, J. Bact. 31: 581, 1936.
3. Moore, H. N., and Kersten, H.: The Inactivation of Encephalitis Virus (St. Louis Type) by Means of Soft X-rays, J. Bact. 33: 625, 1937.
4. Blank, I. H., and Kersten, H.: The Inhibition of Growth of Bacillus Subtilis on a Modified Extract Agar by X-Radiation of the Mediums, J. Bact. 30: 21, 1935.
5. Kelly, J. F.: The X-ray as an Aid in the Treatment of Gas Gangrene, Radiology 20: 296, 1933.
6. Kelly, J. F., and Dowell, D. A.: Present Status of the X-rays as an Aid in the Treatment of Gas Gangrene, J. A. M. A. 107: 1114, 1936.
7. Kelly, J. F., Dowell, D. A., Russum, C. B., and Colien, F. E.: X-ray Treatment in Cases of Gas Gangrene, Radiology. In Press.
8. Lacassagne, A.: Action des rayons K de l'aluminium sur quelques Microbes, Compt. rend. Acad. d. sc. 186: 1316, 1928.
9. Desjardins, A. U.: Radiotherapy (Roentgen Rays; Radium), J. A. M. A. 105: 2153, 1935.
10. Cornell, B. S.: A Chronic Infection With B. Welchii, J. Infect. Dis. 36: 425, 1925.

# THE ROLE OF THE UPPER GASTROINTESTINAL TRACT IN THE ETIOLOGY OF PERNICIOUS ANEMIA\*

## AN EXPERIMENTAL STUDY IN DOGS

W. H. BACHMACH, B.S. AND S. J. FOLLOSON, M.D. CHICAGO, ILL.

AFTER reviewing the literature on the utilization of normal laboratory animals in assaying antipernicious anemia substances, we<sup>1</sup> concluded that no animal has as yet been found to exhibit a uniform or characteristic reaction to such substances. We then decided that the ideal laboratory assay for determining liver potency would be attained only after production of experimental pernicious anemia. The objective of this report is to present our experimental results in attempting to produce this disease in dogs.

### RATIONALE FOR EXPERIMENTAL PROCEDURE

Castle and his associates have developed the most widely accepted theory on the etiology of pernicious anemia. They have shown that in the patient with pernicious anemia there is a deficiency or absence of some factor essential to the formation of the thermostable liver substance. This factor, apparently of enzymic nature, was first believed to originate only in the stomach. Other investigators have shown it to be also present in the duodenum. These observations have explained the failure to produce pernicious anemia, not only after total gastrectomy, but also after exhaustion of the blood forming precursors in these animals by prolonged bleeding.<sup>2</sup>

Dog liver has been shown to be effective in pernicious anemia,<sup>10, 13</sup> but is only one fifth to one fourth as potent as hog liver. Dog stomach<sup>2</sup> and dog gastric juice<sup>8</sup> are apparently inert. These findings suggest that the intrinsic factor is either not present in the dog or is produced elsewhere in the intestinal tract. If the dog has no intrinsic gastric factor, there must be some other way of synthesizing the thermostable liver substance, because we have observed dogs on liver free diets for over two years without the development of pernicious anemia. Another possibility is that the amount of intrinsic factor in the dog's stomach is small and of relative unimportance as compared to the quantity present elsewhere in the upper intestinal tract. This conclusion was the basis for our experiments.

### METHODS

Mongrel dogs of both sexes were kept preoperatively on liberal general diets for two or three weeks. Under ether anesthetic the jejunum was tran-

\*From the Departments of Physiology and Surgery, Northwestern University Medical School, Chicago.

Received for publication April 13, 1938.

TABLE I

RED BLOOD CELL, HEMOGLOBIN, AND HEMATOCRIT DETERMINATIONS ON 31 NORMAL DOGS

RED BLOOD CELLS MILLION	HEMOGLOBIN* PER CENT	HEMATOCRIT
5.70	89	45
5.85	91	47
6.15	100	48
6.20	101	45
6.27	92	45
6.29	102	47
6.17	97	44
6.49	85	41
6.50	99	47
6.53	104	50
6.70	92	46
6.71	98	48
6.74	113	53
6.80	101	47
6.83	95	49
6.87	109	49
6.97	105	46
6.98	106	50
7.05	99	50
7.05	109	53
7.07	97	47
7.10	102	54
7.13	108	51
7.18	108	56
7.43	100	49
7.71	114	56
7.74	99	48
7.91	103	51
8.14	107	55
8.26	97	48
9.34	117	54
Average 6.97	101.3	49

\*Hemoglobin 100 per cent = 14.1 gm.

*Blood Pictures.*—At no time did any of these 7 animals show a blood picture even suggestive of pernicious anemia. Detailed hematologic data on each dog would serve no purpose. They all exhibited a postoperative secondary anemia from which all but one recovered. This one persisting case of anemia, which is of eighteen months' duration, can hardly be attributed to reduction in gastric acidity, because gastric function in 4 of the remaining animals has been equally impaired. Table II summarizes the blood picture of each dog. The blood values in all these animals apparently showed greater fluctuations than customary in animals subjected to other major abdominal interference. Perhaps the instability of the blood regulatory mechanism may be secondary to the extensive surgical intervention. Reticulocyte counts and blood smears made at various intervals were essentially normal.

Biopsies of femoral bone marrow shaft showed nothing microscopically other than perhaps a moderate activity which was relatively insignificant as compared with the hyperplasia seen in gastrectomized dogs, but not suggestive to the slightest degree of pernicious anemia. The femoral bone marrow of Dog 123, sacrificed two years postoperative, appeared grossly almost entirely fatty.

TABLE II

SUMMARY OF POSTOPERATIVE RED BLOOD CELLS, HEMOGLOBIN, AND HEMATOCRIT  
ON DOGS IN THIS SERIES

DOG	RED BLOOD CELLS MILLIONS	HEMOGLOBIN GRAMS	HEMATOCRIT
90	7.16	14.41	51
106	6.93	12.90	47
115	5.88	12.01	41
120	6.17	14.12	47
123	6.98	13.49	47
127	7.73	14.12	47
143	7.35	15.26	46

*Gastrointestinal Studies*—Fluoroscopic and roentgenologic studies of the residual stomach segment showed a gradual increase to practically normal size, despite this 3 of the 7 animals in this series remained almost entirely achlorhydric to alcohol and histamine stimulation as determined by our method of fractional analysis. Our recent study of 28 normal dogs showed an average of 40 clinical units of free acid for the peak secretion to alcohol and 90 clinical units to histamine. In over 300 fractional gastric analyses performed on normal dogs we have never found one refractory to stimulation by alcohol. The three dogs, Nos 120, 127 and 123, gave no free acid response either to alcohol or to the subcutaneous injections of 1 mg. histamine hydrochloride, which justifies our assumption that they are unable to secrete practically any acid. Table III compares the acid secretion of the 7 dogs to the average in 28 normal animals. Perhaps by Neeheles and Scheman's technique,<sup>7</sup> small amounts of free hydrochloric acid might have been demonstrated by constant aspiration.

TABLE III

POSTOPERATIVE GASTRIC ACIDITY VALUES COMPARED TO NORMAL

<i>Response to Alcohol</i>								
TIME HOURS	NORMAL	DOG 90	DOG 106	DOG 115	DOG 120	DOG 123	DOG 127	DOG 143
CLINICAL UNITS FREE ACID								
0.5	28	0	55.0	0	0	0	0	0
1.0	40	17.5	10.0	0	0	0	0	0
1.5	25	0	0	0	0	0	0	0
2.0	13	0	0	0	0	0	0	0
<i>Response to Histamine</i>								
0.5	90	35.0	27.5	0	0	20.0	0	0
1.0	87	32.5	42.5	0	0	0	0	0
1.5	52	0	55.0	0	0	0	0	0
2.0	24	0	0	0	0	0	0	0

The emptying time of a standard meal was proportionate to the size of the stomach. Those dogs with the greatest hypertrophy of the gastric remnant, Nos 90, 106, 115, and 120, had an emptying time which approached normal.

The following are protocols of two dogs representative of the group:

Dog No. 90.—Operated upon Sept. 20, 1935; weight 39 pounds. Following operation the red blood count was 4.71, Hb. 11.3 gm. On March 3, 1936, the acid secretion fifteen minutes after 1 mg. histamine, was 50 clinical units free, 100 clinical units total. There was no response to alcohol. On April 18, the red count was 7.25, Hb. 13 gm. About one year postoperative, Sept. 9, 1936, twenty-four hours after ingestion of 250 gm. of dog food, no residue was found in the stomach; bile was present. Similar absence of residue occurred after this test in the remaining animals of the series. September 25, the dog passed barium sulfate in the stools four hours after its introduction by stomach tube. Beginning November 11, a solution of liver extract equivalent to 15 gm. of fresh liver was given intravenously for five days. Neither this dog nor the others in the series showed any reticulocyte changes. On Feb. 20, 1937, fifteen months postoperative, the emptying time of the standard meal was four hours, ten minutes (normal five-plus hours). On April 4, the response thirty minutes after subcutaneous histamine was 50 clinical units free and 55 clinical units total acid. The Price-Jones curve yielded a mean red cell diameter of  $6.63\ \mu$  (lower normal). The slide showed no evidence of anisocytosis, poikilocytosis, or polychromatophilia. A biopsy of the femoral shaft bone marrow on Dec. 1, 1937, twenty-seven months postoperative, showed scattered areas of very slight activity. Weight at present 42 pounds.

Dog No. 123.—Operated upon Dec. 6, 1935; weight 25 pounds. Recovery was surprisingly rapid. The red cell count on December 11 was 4.58, Hb. 8.6 gm. Until one year later there was no gastric secretion to either alcohol or histamine. On December 24, 1936, the gastric sample showed 20 clinical units free and no combined acid thirty minutes after subcutaneous injection of 1 mg. histamine. On Feb. 20, 1937, the standard meal had left the stomach in three hours and fifteen minutes. On April 3, the mean red cell diameter was  $6.48\ \mu$ , which is slightly smaller than the normal. There was no anisocytosis, poikilocytosis, or polychromatophilia. On November 4, the blood count showed 6.98 million red cells and 13.5 gm. hemoglobin. On Dec. 6, 1937, two years after the operation, the dog was sacrificed, weight 32.3 pounds. Autopsy showed about one-fourth to one-third of the stomach present. The choleodochojejunal junction appeared anatomically reconstructed, with no dilatation of the biliary passages. The femoral bone marrow was grossly and microscopically fatty.

#### COMMENT

The failures of other investigators as well as ourselves to produce experimental pernicious anemia, suggests utilization of an entirely different approach in solution of the problem. Theoretically we should have succeeded if Meulengracht's hypothesis<sup>4</sup> is applicable to the dog. From his experiments on hog tissues he has submitted the hypothesis that the intrinsic factor of Castle originates in the so-called "pyloric gland organ," which includes the pyloric and cardiac glands of the stomach and Brunner's glands in the small intestine. Operative procedures which remove this area should produce pernicious anemia experimentally. Acting upon this suggestion, Petri and co-workers<sup>5</sup> removed the stomach and suprapapillary portion of the duodenum from dogs (in which Brunner's glands are said never to extend beyond the ampulla of Vater), with negative results. In our dogs we removed gastric tissue well beyond the limits of the pyloric glands, as measured by Lim,<sup>3</sup> who also found that the cardiac glands in dogs' stomachs are relatively sparse. We assumed, therefore, that we have removed practically the entire "pyloric gland organ."

Pernicious anemia has not appeared in gastrectomized dogs who have also had extensive enterectomy and pancreatectomy.<sup>14</sup> Schemensky's<sup>12</sup> report on the pernicious anemia properties of the hog colon would perhaps indicate that

the entire gastrointestinal tract is active. These observations support the opinion that the loss of hemopoietic elements from the human subject in pernicious anemia patients may be due to a disturbance or deficiency elsewhere in the body. Vitamin B deficiency studies reported by Miller and Rhoads,<sup>5</sup> and by Wills,<sup>15</sup> appear most significant in this connection. Unfortunately, their work could not be confirmed by Wigodsky, Bussabarger, and Ivy.<sup>14</sup>

## SUMMARY

Seven dogs were observed for a period of over two years following radical subtotal gastrectomy, complete duodenectomy and partial jejunectomy. None of these animals have shown any evidence of pernicious anemia. The pyloric gland organ in the dog is not essential for maintenance of normal dog blood pictures.

## REFERENCES

1. Bachrach, W. H. and Fogelson, S. I. The Effect of Antipernicious Anemia Substances Upon Guinea Pig Reticulocytosis and a Review of the Literature, *J. Lab. & Clin. Med.* 22: 925, 1937.
2. Ivy, A. C., Richter, O., and Kim, M. S. Some Experiments Bearing on the Etiology of Pernicious Anemia, *Am. J. Physiol.* 101: 59, 1933.
3. Lun, R. K. S. The Gastric Mucosa, *Quart. J. Microsc. 66*: 187, 1922.
4. Meulengracht, E. Weitere Untersuchungen über Pylorusdrüsen und Brunnersche Drüsen in ihrer Beziehung zur perniziösen Anämie, *Ztschr. f. klin. Med.* 130: 468, 1936.
5. Miller, D. K., and Rhoads, C. P. The Experimental Production of Loss of Hematopoietic Elements of the Gastric Secretion and the Liver in Swine With Achlorhydria and Anemia, *J. Clin. Investigation* 14: 153, 1935.
6. Mullenix, R. B., Dragstedt, C. A., and Bradley, J. D. Hemoglobin Regeneration in Gastrectomized Dogs, *Am. J. Physiol.* 105: 443, 1933.
7. Neeheles, H., and Scheman, L. Detection of Free Acid in Patients With Suspected Anacidity, *J. A. M. A.* 103: 107, 1934.
8. Palmer, W. L. Quoted by Bussabarger, R. A. A Study of the Effect of Gastrectomy in Immature Dogs, Doctor's Thesis Northwestern University, 1937.
9. Petri, S., Baggild, D., and Ohlsen, A. S. Experimentelle Untersuchungen über gastrogene Anämien (bei Hunden), *Hospitaltid.* 79: 509, 1936.
10. Richter, O., Ivy, A. C., and Meyer, A. L. Study of Dog's Stomach and Liver for Substances Effective in Pernicious Anemia, *Proc. Soc. Exper. Biol. & Med.* 31: 550, 1934.
11. Rosahn, P. D. A New Capillary Hematocrit, *Proc. Soc. Exper. Biol. & Med.* 28: 491, 1931.
12. Schemensl, W. Zur Pathologie der perniziösen Anämie. Therapeutische Erfolge mit Verfütterung getrockneten Schweinegedärmpulvers, *Ztschr. f. klin. Med.* 128: 428, 1935.
13. Strauss, M. B., and Cristle, W. B. Amount of Material Effective in Pernicious Anemia Present in Dog Liver, *Proc. Soc. Exper. Biol. & Med.* 31: 360, 1933.
14. Wigodsky, H. S., Bussabarger, R. A., and Ivy, A. C. Personal communication.
15. Wills, L., and Bilmora, H. Studies in Pernicious Anemia of Pregnancy. Production of Macrocytic Anemia in Monkeys by Deficient Feeding, *Indian J. Med. Research* 20: 391, 1932.

# STUDIES IN CONVULSANT THERAPY\*

## II. THE ROLE OF ALKALINIZATION

STANLEY ROCHELLE DEAN, M.D., NEWTOWN, CONN.

### INTRODUCTION

THE past few years have introduced a new era in the treatment of schizophrenia, the full import of which still remains to be realized. Unlike the haphazard and multifarious methods that have been employed since antiquity to "shock patients into reality," concerted efforts are at last being made to view the problem of irritative or shock therapy in the sober light of scientific investigation.

Sakel's treatment with insulin shock and von Meduna's treatment with metrazol convulsions are the two most important methods in vogue today, and in both of these the reported results have thus far been comparable. Metrazol (pentamethylenetetrazol), however, by virtue of its greater safety and ease of administration, has created widespread interest during the relatively short time that has elapsed since its introduction in this country. Because of its growing importance, it is essential to scrutinize this drug in the utmost detail, not alone from a clinical, but also from a more basic experimental point of view.

For several months our investigations have been directed toward various agents that might be synergistic, antagonistic, or neutral to the convulsant action of metrazol. The present report deals with the effects of diet and the alkaline state.

Friedman,<sup>1</sup> in his earlier work in this country, stressed the importance of thorough alkalization and hydration as an adjunct to convulsant therapy, basing that procedure upon the fact that the alkaline state is tetanic or convulsive in itself and should, therefore, assist in the maintenance of an irritative background in the central nervous system; thus, theoretically, the convulsive threshold would be lowered, less metrazol would be required to produce a paroxysm, and the number of grand mal attacks would be materially increased. He set the minimal fluid intake at two liters per day, and suggested the use of a high alkaline-ash diet supplemented by sufficient alkali drugs to maintain the urinary reaction constantly blue to litmus paper.

These principles are, of course, the converse of those utilized in the treatment of epilepsy, i.e., with ketogenic diets and dehydration, and, theoretically at least, appear to be sound. From a practical viewpoint, however, we found the procedure to be undesirable for the following reasons:

1. High alkaline-ash diets are objectionable to patients and impose an added burden upon the hospital.

\*From the Fairfield State Hospital, Newtown.

Received for publication, April 25, 1938.

2 The simultaneous exhibition of two potent alterative agents (in this case alkalosis and metrazol) makes it more difficult to evaluate results from a scientific point of view than if one agent alone were used

3 Prolonged alkalinization may lead to alkalosis, with varying degrees of toxicity in susceptible individuals. Among the patients described by Ellis,<sup>2</sup> Koehler,<sup>3</sup> Gatewood and his co-workers,<sup>4</sup> and numerous others, as resulting from excessive alkali ingestion, are headache, drowsiness, anorexia, nausea, vomiting, myalgias, nervousness, mental depression, fatigue, faintness, irritability, vertigo, delirium, and tetany.

It seemed to us, therefore, that it would be of decided value to determine just how necessary, if at all, was the role of alkalinization in convulsant therapy.

#### PROCEDURE

The description of metrazol, its mode of administration, and clinical effects have been the subject of another paper. Suffice it to state here that the routine course of treatment is conducted as follows. We begin with the rapid intravenous injection of 3 cc of a 10 per cent solution of the drug, and if no convulsion occurs, increase the succeeding doses by increments of 1 cc every other day until a grand mal seizure results. The convulsant dose having been determined (usually from 3 to 5 cc), it is repeated at the next treatment, and then automatically increased by 0.5 cc at every third injection as long as convulsions occur in an unbroken series, this dosage plan may be indicated numerically as follows, beginning with the initial convulsant dose: 4 4 4½ 5 5 5½ 6 6 6½ 7 7 7½ 8 8, etc.

If, however, a petit mal reaction occurs at any point, the next dose should be increased by one cubic centimeter, bearing in mind the fact that the maximum dose of 15 cc must not be exceeded. Of course, it is not likely that this plan can be invariably followed without deviation in every case, but by adhering to it as rigidly as possible, it is often possible to keep a step in advance of the patient's tolerance, so to speak, and thereby reduce petit mal reactions to a minimum. Then, too, it provides one with a crude yardstick for comparing various situations, for although there is considerable individual variation in the response to metrazol, the use of a standard procedure in hundreds of injections results in a fairly constant ratio of grand mal to petit mal reactions.

In the present instance our study comprised a total of 636 metrazol injections, of which the first 287 were given in conjunction with the alkaline regime, whereas the remaining 349 were administered to patients who received an ordinary house diet without alkalis. For the sake of convenience we may designate the first series, Group A, the second series, Group B.

The high alkaline ash diet used in Group A was prepared from the following prescription:

Protein	71.2 gm
Fat	100.9 gm
Carbohydrate	303.8 gm
Total calories	2,403.0
Excess acid	14.6
Excess base	100.5

\*Calculated for us by Adella Beeuwkes of the University Hospital, Ann Arbor, Mich.



The daily caloric intake, however, was not limited to the above basic requirements, the patient being encouraged to eat as much as he desired. The diet was supplemented by a palatable, effervescent citrate carbonate preparation\* in amounts sufficient to keep the urine constantly alkaline to litmus paper. This alkaline regime was instituted a week before, and then continued throughout the entire course of metrazol therapy, a period of two to three months in duration.

Table I provides a comparison of some of the measurable observations in the two series.

TABLE I

	GROUP A (ALKALI) 287 INJECTIONS	GROUP B (NO ALKALI) 349 INJECTIONS
1. Ratio of grand mal to petit mal reactions	2.8:1.0	2.5:1.0
2. Average initial convulsant dose of metrazol	4 c.c.	4 c.c.
3. Average dose required to produce the twentieth convulsion	9.7 c.c.	9.8 c.c.
4. Average duration of metrazol episode (injection to resting stage)	62 sec.	65 sec.
5. Average weight gain after sixty days of treatment	2.66 kg.	3.42 kg.

## COMMENT AND CONCLUSIONS

Although the observations recorded in Table I are almost self-explanatory, it might be interesting to consider them in some detail. The ratio of grand mal to petit mal reactions, which had been 2.8:1 throughout the first series of injections, dropped slightly to 2.5:1 a week after alkalization and hydration were discontinued, and remained constant at that level throughout the second series. This drop apparently indicates a slightly diminished reactivity of Group B to metrazol, but obviously not enough to be of any practical importance.

Another observation of interest pertains to the initial convulsant dose of metrazol in each series. If alkalization and hydration really lower the convulsive threshold, one should expect this dose to be correspondingly reduced in Group A; yet the averages of several initial convulsions indicate that the minimal dose is exactly the same in either case.

Significant as these two clues may be, a more important consideration, in our opinion, is the average dose required to produce the twentieth convulsion, this number being arbitrarily chosen as representing a late period in the metrazol regime. It is known that a patient's tolerance to metrazol gradually increases with successive injections, and that the effective amount of metrazol must, therefore, be correspondingly increased. Therefore, a comparison of average doses at the late period selected ought to demonstrate convincingly whether or not alkalization and the so-called "irritative background" are effective in reducing tolerance. Reference to Table I definitely indicates that they are not—since the dose in each series is practically the same.

Does the alkaline state augment the duration and severity of the paroxysm? The average duration of the metrazol episode is sixty-two seconds in Group A; sixty-five seconds in Group B; the very slight difference probably falls within the limits of error. Our clinical impression is that there is actually little or no

\*Citrocarbonate (Upjohn).

difference in duration or intensity. True, there is evidence of increased neuromuscular irritability in Group A, for tapping certain areas sometimes produces marked muscular twitching and jeritation, but this phenomenon seems to have no effect upon the metrazol convulsion itself.

It is interesting to note the greater weight gain in Group B. This is not hard to explain when one realizes the average patient's aversion to the alkaline diet, and the fact that anorexia is itself symptomatic of prolonged alkalimiza- tion. It is necessary to point out that this gain in weight is not limited to a sixty day period (arbitrarily selected for convenient comparison), but may go on, in favorable cases, for several weeks or months after the course of metrazol has been completed.

Thus far we have been dealing with measurable phenomena. Equally important to us, though harder to demonstrate to others is our clinical impression. All of us who have followed this investigation have been impressed with the advantages both to the patient and the hospital of the alkaline diet regime. Considering all these factors together, we must conclude that not only are alkalimiza- tion and hydration unnecessary, but definitely undesirable in convulsant therapy.

#### REFERENCES

- 1 Friedman, E. The Irritative Treatment of Schizophrenia. *Am J Psychiat* 94: 755, 1937.
- Irritative Therapy of Schizophrenia. *New York State J Med* 37: 1, 1937.
- 2 Ellis, A. W. M. Quoted in the *Cyclopedia of Medicine*, Philadelphia, 1934, F. A. Davis Co. 1: 313.
- 3 Koehler, A. E. *Ibid*.
- 4 Gatewood, W. L., Giebler, O. H., Muntwyler, E., and Meyers, V. C. *Ibid*.
- 5 Dean, S. R. Studies in Convulsant Therapy. I. Technique and Clinical Phenomena, *J Connecticut M Soc* 2: 325, 1938.

## ABNORMAL UTERINE BLEEDING AS A SYMPTOM IN TYPHOID FEVER\*

CHARLES P. WOFFORD, M.D., CLEVELAND, OHIO, AND DUNCAN G. CALDER, M.D.,  
AND FERDINAND FETTER, M.D., PHILADELPHIA, PA.

**T**HREE patients were admitted to the Philadelphia General Hospital within a relatively short period of time, each suffering with typhoid fever and showing in addition such abnormalities of the menstrual cycle that two of the three were first assigned to the gynecologic wards. In fact, one of the patients was thought to have had a septic abortion for several days until laboratory studies established the true diagnosis. This coincidence led us to investigate the frequency of abnormal uterine bleeding in association with typhoid fever.

There is brief mention of this symptom in the literature. McCrae<sup>1</sup> observes that menstruation usually "ceases during the course of the disease, although at the onset it is not uncommon for it to occur, often with a short interval since the preceding period." However, he found uterine bleeding present in only 11 of 438 women with typhoid fever, and then not in any profuse amounts. Occasional excessive uterine bleeding was described in the hemorrhagic type of typhoid fever, fortunately a very rare form of the disease. Edwards<sup>2</sup> states that "menstruation often appears early at the onset of typhoid" and that there is amenorrhea in some 60 per cent of cases. It is quite generally agreed (McCrae,<sup>1</sup> Edwards,<sup>2</sup> and Hare<sup>3</sup>) that abortion or premature labor is very frequent when pregnancy is complicated by typhoid fever.

It is, of course, well known that various menstrual disorders are associated with many infectious diseases of both the acute and chronic types. However, in view of the paucity of attention called to this symptom in typhoid fever, the following case histories are briefly summarized:

CASE 1.—E. C., white female, aged 23 years. The patient was admitted September 1, 1937, to the gynecologic wards with a history of nausea, vomiting, headache, malaise, generalized aches and pains, together with localized epigastric and lower abdominal pains, dating back eighteen days. Constipation was present until the day before admission, when it was replaced by diarrhea. Uterine bleeding and a recently irregular menstrual cycle accounted for her admission to the gynecologic department. Her menses had begun at the age of 12 years, were always regular, every twenty-eight to thirty days, lasting three days, with a moderate flow and occasional cramps on the first day. Six weeks prior to admission, July 15, her expected period began normally, but lasted six days instead of the usual three. After two days with no bleeding, the bleeding recurred and lasted another week. On August 15, another period began and lasted only two days. On August 28, four days

\*From the Medical Service of Dr. Truman G. Schnabel, Philadelphia General Hospital, Philadelphia.

Read at the Section on General Medicine of the College of Physicians of Philadelphia, Pa., April 25, 1938.

Received for publication, May 7, 1938.

before admission, metrorrhagia again appeared, associated with the passage of numerous clots, some fairly large in size. A steady, small flow continued until her arrival at the hospital. Systemic review, previous medical history, family history, and social history, added nothing of significance, save for one normal pregnancy several years ago.

On physical examination the young woman did not appear acutely ill in spite of a temperature of 104° F. The pulse was 124. No rose spots were visible. The tongue was heavily coated and the pharynx injected. Examination of the cardiovascular and respiratory systems revealed no abnormalities. Pelvic examination showed slight uterine bleeding, a erythematous cervix and vaginal vault, and an external os which admitted one finger. The adnexae were normal. The uterus was retroflexed, movable, but not increased in size nor softened in consistency. No other pertinent physical findings were noted. Uterine bleeding stopped within a few hours after admission, only to recur four days later and last two days, with the passage of numerous clots.

The true diagnosis was reached only after a week during which time she had been treated with blood transfusions and other appropriate measures for a possible septic abortion. On the seventh day after admission—the twenty-fifth day of her disease—she developed rose spots, and the blood culture was reported positive for *B. typhosus*, 12 colonies per 3 cc of blood. The Widal reaction showed an agglutination in a dilution of 1:640 for the H antigen, and 1:320 for the O antigen. It was negative for *paratyphosus A* and *B* and *Brucella abortus*. The leucocyte count was consistent with typhoid fever, 7,200 per c mm on one occasion, and 8,700 per c mm on another, with a differential in the former case of 82 per cent neutrophils, 12 per cent lymphocytes, 4 per cent monocytes, and 2 per cent eosinophiles, and a Schilling index of 40 and a multiple index of 640.

She was transferred to the medical service of Dr. Truman G. Schnabel on September 8, and her subsequent course was uneventful. Her temperature became normal by September 10 and remained so except for a brief elevation five days later when it reached 100° F. During her hospitalization there was nothing further to indicate the possibility of an abortion, and it was obvious that this initial diagnosis was erroneous. She was discharged from the hospital on October 3, 1937. Just before her discharge, October 3, another menstrual period started. This lasted until October 17 and was associated with severe cramps, but no clots. The bleeding each day was more profuse than normal. Again on November 10, a menstrual period began and lasted seven days, with moderate cramps. Otherwise she felt well since leaving the hospital.

CASE 2—A C, negro female, 18 years of age. The patient was admitted August 23, 1937, to the medical service of Dr. Truman G. Schnabel, with symptoms of only three days' duration, namely, headache, nausea, repeated vomiting, anorexia and lower abdominal pain. A nonproductive cough was present. In the systemic review no further points were of interest save her menstrual history. Her menses began when she was 14 years old. They were always regular, every thirty days, lasting two to four days. Her last period began July 25, lasted three days and was normal in all respects. She had had one full term pregnancy, ending in delivery in February, 1936.

Physical examination on admission showed an acutely ill colored girl, with a temperature of 104° F and a pulse of 100. The breath was foul, the tongue was coated, and the tonsils and pharynx injected. Examination of the chest revealed some impairment to percussion in the left lower lobe, with slight suppression of breath sounds. The cardiovascular system was normal. Abdominal palpation elicited generalized tenderness, and there were moderate distention and increased, high pitched peristalsis. Pelvic examination showed a small amount of uterine bleeding, of which the patient was unaware, and a cervix which was lacerated from her previous pregnancy. The uterus was small and slightly anteverted. Pain was elicited on manipulation of the cervix.

The clinical diagnosis of typhoid fever was confirmed by the laboratory three days after admission, with the report of a positive urine culture for *B. typhosus*, and further substantiated two days later by a positive blood culture for the same organism, 2 colonies

per 3 c.c. of blood. The Widal reaction, however, was never positive in a higher dilution than 1:160 in the O antigen, and was negative in the H antigen during her entire stay in the hospital.

Three days after admission, the sixth day of her disease, she began passing numerous clots of blood from the vagina. This was thirty-two days after her last period. On August 28, more than 8 ounces of clotted blood were evacuated at one time. The clots were interspersed with a foul-smelling, reddish-brown vaginal discharge. All bleeding had stopped by September 2, ten days after admission. There was no subsequent bleeding while on the wards. Her clinical course in the hospital was stormy, complicated by an acute suppurative parotitis, which required surgical intervention. She recovered satisfactorily and was discharged in good condition on October 29, 1937.

A follow-up examination on December 7, 1937, found the patient in good physical condition. She had gained over 15 pounds in weight and was symptom free. She stated, however, that she had had no uterine bleeding since September 2, save for slight spotting for one hour on November 28. Pelvic examination failed to reveal any evidences of pregnancy or gross abnormalities.

CASE 3.—M. F., white female, aged 35 years. This patient's illness was ushered in by severe frontal headache and uterine bleeding seven days before admission to the gynecologic wards of the hospital on October 11, 1937. She had been well until that time, but on awakening on the morning of October 4, she had such a severe headache that she fainted on the way to the bathroom. Profuse uterine bleeding made its appearance almost immediately and continued for two days. There was no bleeding thereafter, even during her entire hospital course. Diarrhea, anorexia, and occasional vomiting were also experienced during the week prior to admission. Her menses had begun at the age of 11 years, were always regular every thirty days, lasting four to five days. After missing three periods, she had a self-induced abortion in May, 1937, and had a totally irregular menstrual cycle after that episode, characterized by occasional spotting, occurring every two to three days, filling one pad, but with no normal flow. The patient had four living children and two abortions, the last as mentioned above. No other points in the history were significant.

On admission the patient appeared acutely ill, with a temperature of 104.6° F. and a pulse of 88. She had an intense headache, was sullen but cooperative. The tongue was heavily coated, the breath fetid, and the pharynx injected. The abdomen was slightly distended, and peristalsis was hyperactive. Rose spots were scattered over the abdomen. Pelvic examination was negative. There was no uterine bleeding at that time. The remainder of the physical examination likewise revealed no pertinent findings.

The blood count on admission showed: R.B.C. 4,780,000 per c. mm., Hb. 71 per cent, W.B.C. 6,700 per c. mm., neutrophils 64 per cent, lymphocytes 28 per cent, monocytes 8 per cent, with a Schilling index of 4.3 and a multiple index of 68. Subsequent blood counts showed the same general picture, but with an increasing left shift. A blood culture on October 12 was positive for *B. typhosus*, 4 colonies per 3 c.c. of blood, and the Widal reaction was positive in a dilution of 1:320 for the O antigen and 1:160 for the H antigen. In spite of treatment, which consisted of blood transfusions, parenteral fluids, and other appropriate measures, her subsequent course was a downhill one, complicated by bronchopneumonia and pyelonephritis, and ended with her death on November 2, 1937.

Post-mortem examination revealed myocardial degeneration, bronchopneumonia in the right lower lobe with passive congestion and edema, and a spleen which was the seat of diffuse hyperplasia. There was hyperplasia of the mesenteric lymph nodes. Numerous typhoid ulcers were found in the ileum, cecum, and colon. The liver showed fatty infiltration and parenchymatous degeneration. The latter change was also present in the pancreas. There was lipid depletion of the adrenals, as well as medullary degeneration. The kidneys showed bilateral pyelonephritis. There was also an ureteritis and hemorrhagic cystitis. The uterus was small, firm, and in normal position. The endometrium was slightly thickened and bloody. No evidence of placental tissue was found on either gross or microscopic examination.

## COMMENT AND SUMMARY

Considering the fact that each of these three patients showed irregularities of the menstrual cycle during her illness, it seems appropriate to stress this symptom as one which may be found at the inception or during the course of typhoid fever. The irregularity was different in each case. In one it took the form of prolonged excessive metrorrhagia with the passage of numerous clots during the first and second weeks of her disease. In the second patient the symptoms were those of menorrhagia, together with a change in the character of the menstrual flow, which became foul smelling reddish brown, and occasionally interspersed with clots. In the first case the menstrual cycle had been disrupted by an abortion five months previously but her typhoid fever was ushered in by unusually profuse uterine bleeding.

We believe that the excessive uterine bleeding in these patients was simply part of the disease, and that it does not represent the extremely rare hemorrhagic type of typhoid fever mentioned by Osler and Medlar.<sup>1</sup> Unfortunately, no bleeding or clotting times or platelet counts were done on these patients. The absence of other hemorrhagic phenomena and the subsidence of the bleeding, however, indicate that a blood dyscrasia was not present. The importance of the symptom lies in the possibility of its confusing the diagnosis, as shown strikingly in the first case cited. This error might be avoided in similar instances if the possibility of menorrhagia and metrorrhagia in typhoid fever is kept in mind. It is our opinion that abnormal uterine bleeding in typhoid fever is encountered more frequently than the brief mention in the literature would indicate.

## REFERENCES

- 1 McCrae, Thomas. Osler's Modern Medicine ed 2 Philadelphia 1925 Lea & Febiger, pp 133, 144
- 2 Edwards, A. R. Principles and Practice of Medicine ed 2 Philadelphia, 1916 Lea & Febiger, p 40
- 3 Hare, H. A. Practice of Medicine ed 2 Philadelphia 1907 Lea Brothers & Co, p 43

## DISSECTING ANEURYSMS OF AORTA\*

HERBERT J. SCHATTENBERG, M.D., AND JOSEPH ZISKIND, M.D., NEW ORLEANS, LA.

A DISSECTING aneurysm usually begins as a tear through the inner layers of a vessel wall, dissects between its layers, and forms a longitudinal and circular cavity. It is, however, believed by some writers, namely, Tyson,<sup>1</sup> Babes and Mironescu,<sup>2</sup> Whitman and Stein,<sup>3</sup> and Krukenberg,<sup>4</sup> that a tear in the intima is not a necessary part in the formation of dissecting aneurysms. They hold that it begins with a rupture of the vasa vasorum into a weakened media with the formation of a hematoma that splits the fibers of the media. These observers, therefore, believe that when a tear in the intima occurs, it is most likely secondary to the formation of the dissecting aneurysm.

In almost all cases the vessel involved is the aorta, but dissecting aneurysms can form in smaller arteries. This aneurysm may perforate to the exterior by tearing through its outer wall, or it may less frequently rupture back into the lumen of the vessel. In the latter case recovery may take place because of the formation of a canal lined by endothelium, through which the blood will circulate. This latter result does not invariably prevent rupture to the exterior. It is, however, very important in the healing process of dissecting aneurysms. It is aided by the fact that the canal passes through tissues that are well supplied with blood vessels, especially on the outer aspect from which organization can take place. Very rarely the blood within the aneurysm will organize, and the channel will be completely obliterated.

Dissecting aneurysm is a relatively rare condition. Walker and Walker<sup>5</sup> found an incidence of one rupture in 2,500 necropsies, and Ames and Townsend<sup>6</sup> found this condition once in 500 autopsies. Weiss<sup>7</sup> has found a ratio of 1:320 in adults.

This disease is usually found in individuals above the age of 40, and the relative incidence increases with age. However, instances of rupture may occur at any age. Klotz and Simpson<sup>8</sup> collected 42 cases of spontaneous rupture of the aorta in individuals under 40. Of these, 2 cases occurred between the ages of 1 and 10, 7 were found between 11 and 20, 20 between 21 and 30, and 13 between 31 and 40. It is also quite interesting to note that it occurs twice as often in males as in females.

The intimal tear may be longitudinal, oblique, or angular, and in some cases has been found to be multiple. It is often transverse and irregular, and measures from 1.5 cm. to 2 cm. in length. This tear in many cases lies about 1.5 cm. above the aortic valve ring. In about 70 per cent of cases the intimal

\*From the Departments of Pathology, Tulane University School of Medicine and the State Charity Hospital of Louisiana.

Received for publication, May 9, 1938.

tear is in the ascending aorta. The remaining 30 per cent is found in the transverse and descending aorta, with more cases in the transverse portion. The dissecting plane is almost always between the middle and outer thirds of the media and involves approximately one half to two thirds of the entire circumference. The length of the sac varies from a few centimeters to almost the entire length of the aorta.

In most cases death is due to secondary rupture of the aorta. Approximately 70 per cent of cases rupture into the pericardial sac and the remainder rupture into the left pleural cavity, the mediastinum, the right pleural cavity, the abdominal cavity, or the retroperitoneal tissue in that approximate order of frequency.

Since the first description of a dissecting aneurysm by Nicholls<sup>9</sup> in 1762 the pathogenesis of this condition has been in dispute. Various theories have arisen which may be classified under four headings, namely, mechanical, inflammatory, degenerative, and congenital.

*Mechanical*—Some authors such as Bostrom<sup>10</sup>, Busse<sup>11</sup>, Oppenheim<sup>12</sup> and Samson<sup>13</sup> believe that a definite external trauma can be frequently elicited in history.

Bostrom<sup>10</sup> maintains that a mechanical trauma is the only factor in the formation of both the primary and secondary ruptures. Rindfleisch<sup>14</sup> claims that certain bands pass directly from the main pulmonary artery to the aorta and by holding these together about 1 cm. from the origin of the vessels and by the ligamentum arteriosum one can determine the usual sites of the primary tears.

It has been generally agreed upon by many observers that forces which cause a sudden cardiovascular strain and increase in blood pressure will not injure the normal aorta. Many believe, however, that such forces may cause the formation of a dissecting aneurysm in persons having congenital cardiovascular anomalies.<sup>15, 16</sup> Oppenheim<sup>12</sup> observed that rupture of the aorta will not occur until a pressure of approximately 3 000 mm. of mercury is reached. This is about twenty times that of the normal pressure.

Samson<sup>13</sup> states that traumatic aneurysms are practically all dissecting. He believes that this type is on the increase because of the more frequent number of industrial and automobile accidents and that they are formed at the time of the accident or immediately following it. The initial tear is located at or near the attachment of the obliterated ductus botalli, or just above the aortic valves on the posterior part of the wall of the aorta. Samson<sup>13</sup> argues further that traumatic dissecting aneurysms may occur in a healthy aorta, providing the causative force is severe enough and that under certain conditions the healthy aorta may be torn completely across.

Weiss<sup>7</sup> claims that the majority of traumatic ruptures of the aorta are not the dissecting type, but that the sudden and severe external force exerted on the noncompressible column of blood will cause a complete tear of the vessel.



Shennan<sup>17</sup> is in agreement with this latter view, that an external force causes complete rupture of the aorta. He does not agree that mechanical injuries, whether external or internal, are very important factors. He does believe, however, that a sudden increase of blood pressure, usually caused by a physical or mental strain, is important only when there is disease of the vessel wall. Far more important to this observer<sup>18</sup> than the increase of the systolic pressure is the abrupt diastolic recoil. In most of Shennan's cases hypertension associated with an hypertrophied left ventricle was present, but in about one-fifth of the reported cases no hypertension was noted.

*Inflammatory.*—Rokitansky<sup>19</sup> appears to be the first to have considered the inflammatory theory. He stated that in one form of dissecting aneurysm the adventitia is easily separated from the media as a thick, vascular layer in a state of chronic inflammation. However, later authors who think that inflammation is a causal factor in dissecting aneurysms believe that it is the media that is involved. To Köster<sup>20</sup> is usually given the credit for the inflammatory theory. His view is that a mesarteritis extends from the adventitia along the vasa vasorum and that this causes fibrosis and destruction of the muscularis and elastica. This weakens the media and allows for rupture when the blood pressure is raised.

Dabes and Mironescu<sup>2</sup> have described degenerative changes with infiltrating cells and with new vessels which, by rupture, will form small hemorrhages in the media. These, therefore, do not arise from the lumen of the artery. To this process they have given the name of "dissecting aortitis." The findings have been corroborated by Krukenberg,<sup>4</sup> Tyson,<sup>1</sup> Whitman and Stein,<sup>3</sup> and others.<sup>21-23</sup> These observers, therefore, concluded that a dissecting aneurysm may form in the absence of a tear of the intima.

Certain specific diseases, such as syphilis and rheumatic fever, have occasionally been considered as being important in the causation of dissecting aneurysm. Syphilis, however, does not appear to be a factor in their production. Klotz and Simpson<sup>8</sup> state that the very nature of the syphilitic process with its gumma and fibrosis does not allow for dissection. In fact, they believe that the granulomatous inflammatory invasion of the arterial wall will tend to hold the neighboring laminae more closely together, so that the wall may be less readily split into its anatomic layers than normally.

Shennan<sup>17</sup> claims that there is some resemblance of the medial and adventitial reactive changes to those described in rheumatic aortitis. It, therefore, seems possible to him that in this disease, degenerative atrophic and acute necrotic changes may be produced in the media which are similar to those caused by other toxins.

*Degenerative.*—According to Virchow and a number of other pathologists,<sup>24, 25</sup> dissecting aneurysm was thought to be formed by the dissection of blood through the base of an atheromatous ulcer or its border into the layers between the media and intima or between the media and adventitia. This is probably true for a small number of cases, but it is in the ascending aorta, where the aorta is relatively immune to arteriosclerosis, that most primary ruptures are found.

Peacock<sup>26</sup> believed that degenerative changes in the aortic wall were important especially when they involved the media. The importance of microscopic interruptions or "faults" in the media was emphasized by Shennan and Pirie<sup>18</sup>. These were associated with atrophic changes partial or complete disappearance of muscle fibers in large areas of the media and degenerative changes in the connective tissue and elastica. These findings were also noted by Moriam<sup>27</sup>.

Gsell<sup>28</sup> found that the media had undergone noninflammatory necroses in his cases. He thought that these lesions were similar to those described by Wiesel<sup>29</sup> who had observed peculiar focal necroses in the vessel walls of young individuals dying from acute infections.

Erdheim,<sup>30</sup> in his study of dissecting aneurysms found lesions in the aorta which were similar to those described by Gsell. He observed small areas of necrosis and peculiar hyalinized vacuoles in the media beyond the region of the rupture. The elastic, muscular and connective tissue elements were involved. No inflammatory reaction and no reparative process were noted. These necroses were most numerous in the outer two thirds of the media. This entire destructive process in the media caused necrosis of the cellular elements, and this disintegrated material was absorbed without local tissue reaction. These areas were then filled with an albuminous fluid which then formed the picture of peculiar cystic spaces between elastic fibers. This author felt that the process of necrosis and that of mucoid degeneration were different. This peculiar medial degeneration was also found more frequently in older individuals, aside from those cases having dissecting aneurysms. It was either diffuse or patchy. As to the etiology of these changes, Erdheim is unable to say, but he believed that the action of toxins is highly important. Klotz and Simpson<sup>8</sup> and Moritz<sup>31</sup> in later studies have found changes in the aorta similar to those described by Erdheim.

*Congenital*—Many cases of dissecting aneurysm in young persons have some congenital changes in the aorta.<sup>1, 16</sup> The most important are coarctation and dilatation of the aorta, with thinning of the wall. Other congenital factors are a thymicolympathic constitution with aortic and cardiac hypoplasia and aortic stenosis.

The two cases here reported show changes in the media of the aorta similar to those described by Erdheim.

*CASE 1*—A D, a colored male, aged 23 years was admitted to the hospital on April 23, 1933, with complaints of cough, pain in the right side, difficulty in breathing, and swelling of the legs. The patient stated that he had had gonorrhea, and "pimples" on the meatus of the urethra of three weeks' duration about a year and a half previously. He denied having a chancre. He had pneumonia in January, 1933, and was confined to bed for nineteen days, after which the least exertion produced dyspnea. The following month he was forced to go to bed again because of dyspnea, cough, and edema of the lower extremities, and severe pain in the right side of the abdomen. Subsequent to the attack of pneumonia and the sequelae, the patient never regained his former health, spending most of his time in bed.

Physical examination revealed a fairly well developed and nourished colored male, with edema of the entire body, who appeared critically ill. Ascites also appeared to be present. The chest was of the asthenic type and breathing was labored. The lungs showed impairment

of resonance at right base posteriorly. In both bases posteriorly, crepitant and subcrepitant râles were heard. The heart was markedly enlarged. The right border was one inch to the right of the sternal margin in fourth right interspace and the left margin extended out to the left axillary line. There was an intense thrill during diastole which was felt over the entire precordium. A loud high pitched diastolic murmur was heard over the entire precordial area. The pulmonic second sound was extremely loud and had a metallic quality. The blood pressure was 140/40. The Wassermann reaction was negative. The urinalysis showed a moderate amount of albumin and a few hyaline and granular casts. The patient died shortly after admission.

*Autopsy:* The body was that of a well-developed well-nourished negro male. The skin was smooth and tense, due to the edematous condition of entire body. The pupils measured 4 mm. and were equal. No penile scars were noted. The pleural cavities contained approximately one liter of blood-tinged fluid. The left lung was adherent to the diaphragm below.

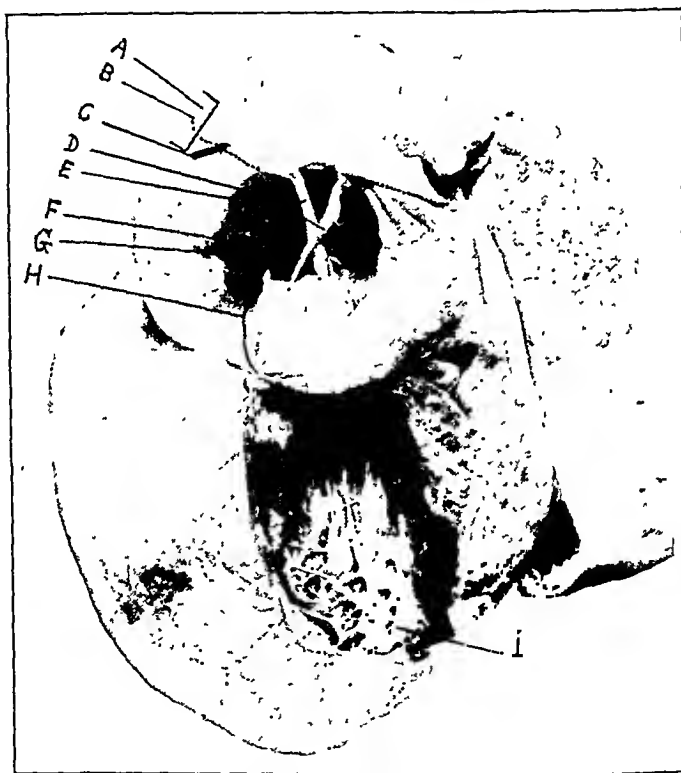


Fig. 1.—Photograph showing dissecting aortic aneurysm presenting the following details: A, Area selected for histologic section, upper portion of which contains all three layers of aortic wall and lower portion which contains only adventitia and part of media (see Fig. 2). B, Dotted line represents course of tear which involved almost entire circumference of vessel. C, Probe indicating minute intimal depression and tear which permitted blood to extravasate from aorta into aortic wall, with subsequent splitting of media into two separate portions. D, Aneurysmal dilatation, wall of which consists of adventitia, outer portion of media and newly formed connective tissue replacing intima and greater part of media. E, Strips consisting of intima and inner portion of media forming typical "X." These structures connect the upper normal portion of aorta with the lower retracted portion "H." F, Probe inserted in small 2 mm. tear in adventitia and outer part of media. It is at this point that hemorrhage took place into the pericardial cavity. G, Tubing inserted to show normal passage from left ventricle through aortic valve and conical structure "H" into aneurysmal dilatation "D." H, Conical projection, 2 cm. in height and 1.5 cm. in diameter, consisting of intima and inner portion of media attached to aortic ring below, only remaining part of proximal portion of aorta not dilated. I, Left ventricular cavity.

The right lung showed numerous adhesions to the ribs anteriorly. On section both lungs were markedly congested. The liver weighed 1,860 gm. The cut surface had a typical "nutmeg" appearance. The spleen weighed 250 gm. and revealed marked congestion. The intestines, stomach, pancreas, adrenals, and kidneys showed considerable congestion.

The pericardial sac was greatly distended and filled a good portion of the left chest cavity. On opening the sac, 970 cc of clotted blood was found.

The heart had a marked increase in the amount of subpericardial fat and weighed 250 gm. The pulmonic valve measured 6 cm, the mitral valve 10 cm, and the tricuspid valve 12 cm. The aortic valve was not opened in order that the relationship of the pericardial hemorrhage to this area might be studied. The left ventricular wall measured 1.5 cm, and the right measured 0.5 cm.

**Aorta.** Externally for a distance of 5 cm the proximal portion of the ascending aorta appeared dilated. Upon opening this dilated structure it was found that the vessel wall in this area had been broken by a dissecting aneurysm. Apparently the primary break had its beginning as indicated by *C* shown in Fig 1. This opening led into the media, but did not penetrate through the aortic wall. It was through this small aperture that blood from the aorta proper infiltrated between the medial layers dissecting toward the adventitia and left the greater part of the elastic structure to the luminal side of the vessel. Blood evidently, traversed the wall, and the entire conical protrusion at *H* was torn loose with the exception of the two strips in an X line arrangement which continued intact. An almost complete circular tear, therefore, involving the intima and a portion of the media occurred at this primary injury. This break apparently weakened the vessel at this point, with a resultant bulging or aneurysmal formation of the remaining unbroken portion of media and



Fig 2—Section of area 1 shown in Fig 1 (low power). *J* Point at which primary tear and dissection took place. The inner media and intima were torn loose at this place to form the conelike protrusion *H* described in Fig 1. *A* Shows normal intact intima. *L* Showing appearance of media a short distance distal to tear. *M* Adventitia. *N* Adventitia. *O* Shows marked proliferation of newly formed edematous connective tissue lying upon remaining portion of media. This newly formed connective tissue constituted the inner lining of the dissecting aneurysm. *O*, Outer portion of media forming a dark line extending through section and continuous with outer portion of *L*.

adventitia. This bulging did not include the aortic ring which remained intact within certain portions of the inner aortic coats (intima and part of media). From the aortic ring these coats protruded upward into the dilated aorta for a distance of 2 cm in a conical form. From the ends of this conelike protrusion, the two stripped portions of the aortic wall connected in an X form with the unbroken aorta above, as shown by *E* (Fig 1). A careful examination for a possible rupture of the remaining outer portion of vessel wall was made and a short linear tear, 0.5 cm in length, was found as indicated by *F*. It was at this point that hemorrhage took place into the pericardial cavity, causing the sudden death of the patient.

A further examination of the aorta extending from the distal portion of the aneurysmal formation down to and including the bifurcation of iliacs, showed only an occasional yellowish elevated area. These lesions were located especially in the thoracic aorta near the openings of the intercostal arteries.

**CASE 2**—This specimen of a dissecting aneurysm was received by us through the courtesy of Dr J E Robinson of Temple Texas. A F, a white male, 56 years of age, was admitted to the hospital with a history of a tearing pain in the chest. This occurred immediately following a meal, and he gradually went into shock. He was treated with morphine for his

pain but died suddenly, twelve hours after his admission. His blood Wassermann was negative, and his past history revealed that he suffered from "rheumatism" and "stomach trouble" for a number of years.

*Autopsy:* The body was that of a well-developed and well-nourished white male, weighing 210 pounds and measuring 61 inches in length. The heart was surrounded by a large

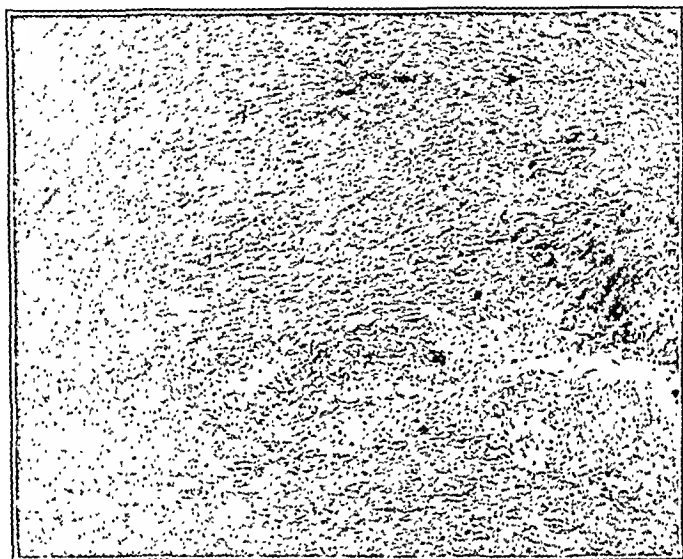


Fig. 3.—An area of medial degeneration with some evidence of healing by fibroblasts and cyst formation.

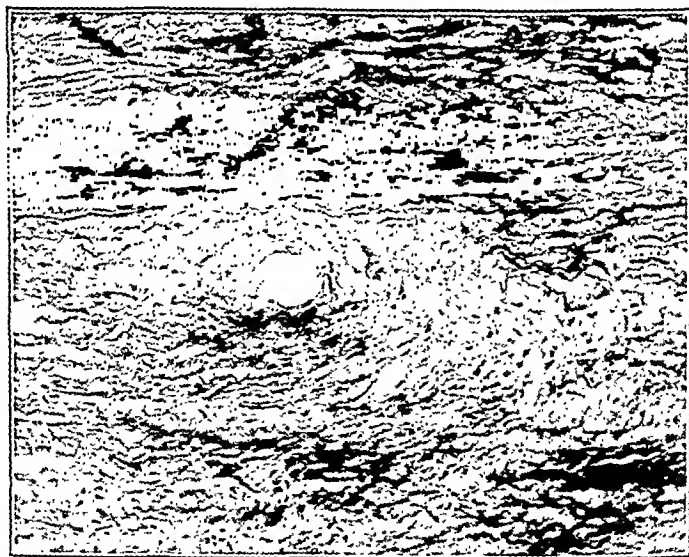


Fig. 4.—High power view showing cystic area and spaces between elastic fibers containing a mucinlike substance. Considerable destruction of elastic lamellae is also present.

amount of freshly clotted blood. The heart was enlarged and weighed 460 gm. A moderate amount of fat was present in the subepicardium. The valves were essentially normal. The left ventricular wall measured 2.2 cm. in width. The myocardium was firm, reddish brown in color, and showed no areas of fibrosis. The coronary arteries were patent and showed an occasional yellow atheromatous plaque.

*Aorta:* A transverse tear, 3 cm. long, was noted through the intima and media about 2 cm. above the aortic valves. The edges of the tear were sharp and a dissecting aneurysm began here and undermined its way for about 7 cm. above the tear and downward into the pericardial cavity through a laceration, 0.7 cm. in length.

*Microscopic Findings:* The histopathologic findings were similar in both cases and will be described together. In Case 1, the lesions were most pronounced in the ascending aorta while in Case 2, they were seen throughout the vessel.

The intima in Case 1 in the region of the tear was normal. The aneurysmal wall showed newly formed connective tissue (Fig. 2), which replaced the original intima and inner media. The intima in Case 2 near the tear showed a slight increase in the subendothelial connective tissue but otherwise the changes were minimal.

The most characteristic lesion consisted of patchy or diffuse necroses in the media. These were found distributed in a general manner, but were most commonly seen in the region of the junction of the middle and outer thirds of the media. No exudative reaction was seen in the neighborhood of these necrotic areas, but some defects were seen undergoing repair by a slight fibroblastic proliferation (Fig. 3).

The muscle fibers and elastic laminae were involved in this process, but the destruction of the former structures was more marked. The muscle fibers showed altered nuclei, fragments of nuclei, or had undergone complete dissolution. Clefts or cysts filled with a mucinlike material were, therefore, seen between elastic fibers (Fig. 4). Generally this mucinlike substance could occasionally be seen between the muscle and elastic fibers without cystic formation. With the increase of this mucinlike material there was a decrease in the number of interlamellar muscle and connective tissue cells. At times the elastic fibers were damaged, as evidenced by fragmentation, irregular thickenings, and loss of normal staining reaction. Occasionally these were completely destroyed. No unusual fatty changes could be seen in the media.

Very little compensatory adventitial thickening was noted in either case. The vasa vasorum in Case 2 showed occasional moderate intimal proliferation, without perivascular infiltration.

#### DISCUSSION

In both cases there were lesions in the media of aorta, especially in the region of the rupture, which were thought to be responsible for the tear in the weakened wall.

The microscopic changes in the media noted in these cases are similar to those described by Gsell and Erdheim. This consists of a peculiar degeneration of the media not associated with a significant intimal or inflammatory reaction. With the degeneration of muscle, connective tissue, and occasional elastic fibers, cystic areas were formed filled with a mucinlike substance. The medial necrosis was most marked in the region of rupture, but was also seen in other parts of the aorta.

The frequency of tear in the first portion of the aorta is probably due to the medial degeneration which occurs with the greatest intensity in this region. With age, there is a tendency for the lesion to become more widely spread throughout the aorta, and it has been suggested by Moritz<sup>11</sup> that the disease is involutional in character.

No definite evidence has been given as to the manner in which the medial necroses occur. Most observers agree that these lesions do not arise because of the disturbance of nutrition due to disease of the vasa vasorum. It appears most likely that these degenerations are due to the action of toxins. These toxins may be bacterial, exogenous, such as nicotine or adrenalin, or meta-

bolic. There is also the likelihood that certain dietary deficiencies may be important factors in the production of this lesion.

### SUMMARY

Two cases of dissecting aneurysm are reported.

The aorta in each case showed cystic necrosis of media, without evidence of inflammation.

It is believed that these changes in the media were the underlying process in the formation of the dissecting aneurysms.

The etiology for this degeneration remains obscure, but dietary deficiencies coupled with the action of toxins may be important factors.

### REFERENCES

1. Tyson, M. D.: Dissecting Aneurysms, *Am. J. Path.* 7: 581, 1931.
2. Babes, V., and Mironescu, T.: Ueber dissezierende Arteriitis und Aneurysma dissecans, *Beitr. z. path. Anat. u. z. allg. Path.* 48: 221, 1910.
3. Whitman, R. G., and Stein, H. B.: A Contribution to the Pathogenesis of Dissecting Aneurysms; a Case of Dissecting Mesoaortitis (Babes and Mironescu) Without Dissecting Aneurysm, *J. M. Res.* 44: 579, 1924.
4. Krukenberg, Ernst: Beiträge zur Frage des Aneurysma dissecans, *Beitr. z. path. Anat. u. z. allg. Path.* 57: 329, 1920.
5. Walker, C., and Walker, L.: Sudden Detachment of Aortic Intima (So-called Dissecting Aneurysm), *Brit. M. J.* 2: 200, 1919.
6. Ames, D., and Townsend, W. G.: Spontaneous Rupture of the Aorta Exclusive of Ruptured Aneurysms; With an Analysis of 50 Cases, *Maryland M. J.* 37: 199, 1897.
7. Weiss, Soma: Clinical Course of Spontaneous Dissecting Aneurysm of Aorta, *M. Clin. North America* 18: 1117, 1935.
8. Klotz, O., and Simpson, W.: Spontaneous Rupture of Aorta, *Am. J. M. Sc.* 184: 455, 1932.
9. Nicholls, F.: Observations Concerning the Body of His Late Majesty, George II, *Phil. Tr. Roy. Soc. London* 52: 265, 1762.
10. Boström, E.: Das Geheilte Aneurysma Dissecans, *Deutsche Arch. f. klin. Med.* 42: 1, 1888.
11. Busse, O.: Ueber Zerreibungen und traumatische Aneurysmen der Aorta, *Virchows Arch. f. path. Anat.* 183: 440, 1906.
12. Oppenheim, F.: Gibt es eine Spontanruptur der gesunden Aorta und wie kommt sie zustande? *München med. Wchnschr.* 65: 1234, 1918.
13. Samson, Paul C.: Dissecting Aneurysms of Aorta, Including Traumatic Type, *Ann. Int. Med.* 5: 117, 1931.
14. Rindfleisch, E.: Zur Entstehung und Heilung des Aneurysma dissecans Aortae, *Virchows Arch. f. path. Anat.* 131: 374, 1893.
15. Marine, D.: Status Lymphaticus, *Arch. Path.* 5: 661, 1928.
16. Hamilton, W. F. (Montreal), and Abbott, M. E.: Coarctation of Aorta of Adult Type; Statistical Study and Historical Retrospect of 200 Recorded Cases With Autopsy, of Stenosis or Obliteration of Descending Arch in Subjects Above Age of 2 Years, *Am. Heart J.* 3: 381, 1928.
17. Shennan, T.: Dissecting Aneurysms, Medical Research Council, Special Report Series No. 193, His Majesty's Stationery Office, 1934.
18. Shennan, T., and Pirie, J. H. H.: The Etiology of Dissecting Aneurysm, *Brit. M. J.* 2: 1287, 1912.
19. Von Rokitsansky, C.: *Pathologie Anatomie*, Syd. Soc., London 4: 313, 1852.
20. Köster, E.: Ueber die Entstehung der spontanen Aneurysmen u. d. Chronische Mesarteriitis, *Berl. klin. Wchnschr.* 12: 322, 1875.
21. Kellogg, F., and Heald, A. H.: Dissecting Aneurysm of Aorta; Report of Case Diagnosed During Life, With Pathologic Study, *J. A. M. A.* 100: 1157, 1933.
22. Crane, M. P.: Medical Papers, Christians Birthday Volume, Baltimore, 1936, Waverly Press, p. 142.
23. Lawrence, John H.: Clinical Symptoms and Signs of Dissecting Aneurysm of Aorta, With Report of Case Diagnosed During Life, *Internat. Clin.* 2: 122, 1935.
24. Glendy, R. E., Castleman, B., and White, P. D.: Dissecting Aneurysm of Aorta; Clinical and Anatomical Analysis of 19 Cases, 13 Acute With Notes on Differential Diagnosis, *Am. Heart J.* 13: 129, 1937.

25. MacCallum, W. G.: Dissecting Aneurysm, *Bull Johns Hopkins Hosp* 20: 9, 1909
26. Peacock, T. B.: Report on Cases of Dissecting Aneurysms, *Tr Path Soc London* 14: 87, 1863.
27. Moriani, G.: Ueber einen Fall von Aneurysma dissecans Aortae mit besonderer Berücksichtigung frischer Rupturen der Aortenmedia, *Virchows Arch f. path. Anat* 202: 283, 1910
28. Gsell, O.: Wandnekrosen der Aorta als selbständige Erkrankung und ihre beziehung zur spontanruptur, *Arch f path Anat.* 270: 1, 1928
29. Wiesel, J.: Die Erkrankungen Arterieller Gefässe im Verlaufe akuter Infektionen 3. Teil. Die akute herdförmige Mesarteritis der Koronararterien und ihre Folgezustände, *Ztschr f Heilk* 28: 69, 1907
30. Erdheim, J.: Medionecrosis aortae idiopathica cystica, *Virchows Arch f path Anat.* 276: 187, 1930
31. Moritz, A. R.: Medionecrosis aortae idiopathica cystica, *Am J Path* 8: 717, 1932

## A STANDARDIZED PROCEDURE FOR THE STUDY OF COAGULATION REACTIONS (IN VITRO)\*

JOHN H. FERGUSON, M.D., ANN ARBOR, MICH

OWING to paucity of knowledge concerning the exact physicochemical nature of the reagents, any investigation of coagulation reactions is rudely dependent upon the particular set of conditions under which the individual experimenter operates. Indeed, his results can often be evaluated only by a full consideration of the limitations imposed by these conditions. The particular advantages claimed for a standardized procedure are (1) control of known variables, (2) a point of departure for the study of physicochemical factors, e.g., temperature, pH, dilution, etc., and (3) a sufficient uniformity of the individual reagents to attempt to relate each of them quantitatively to the coagulation time of the test systems. Several years' experience with a variety of techniques has resulted in the elaboration of the following methods, herewith examined for the degree to which they may claim to serve as a *standardized* procedure for a biochemical approach to coagulation problems

### EXPERIMENTAL TECHNIQUE

*Cell-Free (Platelet-Free) Plasma*—A medium sized, preferably fasting, dog is anesthetized by intraperitoneal barbiturate (e.g., 0.7 cc per kg of 5 per cent pentobarbital sodium). The animal, of course, is to be sacrificed. Blood is collected, with negligible tissue damage, via a paraffined cannula in the femoral artery, the first few cubic centimeters being discarded and the remainder received into one-tenth volume of isotonic (3.8 per cent) trisodium citrate in paraffined centrifuge bottles (250 cc capacity). Preliminary centrifugalization yields a turbid plasma containing platelets, some white cells, and usually a trace of red cells. More rapid and prolonged recentrifugalization in tubes (50 cc capacity) gives a clear plasma, with a certain opalescence varying with the degree of lipemia. In order to remove all traces of platelets, etc., it is

\*From the Pharmacological Laboratory, School of Medicine, University of Michigan Ann Arbor



recommended that the twice-centrifugalized plasma be submitted to rapid filtration through a Berkefeld (V), or similar, filter. The filtrate is chilled and kept in the icebox.

*Prothrombin.*—*Fresh* Berkefeld-filtered plasma is defibrinated by warming cautiously in water bath to 54°-56° C. for two minutes, and then filtering through coarse filter paper. Prothrombin papers are prepared from 5 c.c. quantities of the filtrate by the acetone method of Howell.<sup>1, 16</sup> The method consists simply in precipitation with an equal volume of acetone, immediate collection of the precipitate on a filter paper in a Büchner funnel, rapidly washing with about 20 c.c. of ether, and drying as quickly as possible in an air current (jet or fan). The dry papers may be kept indefinitely and a sufficient supply may be made from a single animal to last for weeks or months. Prothrombin solutions of desired strength are prepared by macerating the cut-up papers for several hours with a suitable volume of distilled water containing a drop or two of 0.5 per cent sodium bicarbonate, and subsequently filtering.

*Prothrombin-Poor Plasma.*—Both the magnesium hydroxide<sup>13, 25</sup> and aluminum hydroxide<sup>23</sup> adsorption methods have proved efficacious in our experience, and the following technique is selected from a number of empirical variations tested. One hundred cubic centimeters of 10 per cent aluminum chloride are precipitated with one-half volume of 5 per cent ammonia. The precipitate is removed by centrifugalization and resuspended in distilled water. Some three to five repetitions of the washing and recentrifugalization are necessary before the preparation is sufficiently free from traces of ammonia. About 50 c.c. of distilled water are used to make the final suspension of "alumina gel." When shaken with 100 to 120 c.c. of Berkefeld plasma at intervals during a two-hour period at icebox temperature, complete, or, more often, nearly complete, adsorption of the prothrombin is effected without serious loss of fibrinogen. High speed centrifugalization is preferred for recovery of the plasma, although slow filtration through coarse filter paper is often satisfactory. It is important to restore the pH of the recovered plasma to approximately 7.5 by adding N/1 hydrochloric acid, drop by drop, with continuous stirring, until a test sample gives the correct color (orange pink) with phenol red indicator.

*Prothrombin-Free Fibrinogen.*—The fibrinogen is precipitated from the adsorbed plasma by treatment with one-third volume of cold saturated ammonium sulfate, the precipitate being recovered by centrifugalization and redissolved in distilled water, or 0.9 per cent sodium chloride, with the addition of sufficient dilute alkali (sodium bicarbonate) to restore to pH 7.5 (phenol red indicator). It is advisable to filter at this stage and then to repeat the "salting out" once or twice. The fibrinogen solution is the one reagent which must be prepared freshly, since no satisfactory method is known for keeping it in stable form.\* The present preparation seldom fails to give a solution (about one-half to three-fourths of the original plasma volume) which is sufficiently stable at icebox temperatures for several days.

\*A method based on high-vacuum desiccation at low temperatures is at present under investigation.

**Cephalin**—The following method of preparing pure cephalin from brain tissue embodies the original technique of Thudichum<sup>17</sup> and the steps to insure the absence of non-amino nitrogen devised by Levene and Rolf.<sup>18</sup> Freshly hashed brain (hog, calf, sheep), freed, of course, from pia mater, etc., is not dried but directly extracted with three lots of 95 per cent alcohol followed by two lots of ether. The alcohol and ether extracts are concentrated separately (vacuum distillation—filter pump) and the "protogens" removed from the cooled concentrates before mixing. The mixed concentrates are further evaporated to a viscid watery magma. This is extracted three times with acetone and once with cold absolute alcohol. The residue is exhausted with ether, and the cephalin precipitated from ethereal solution with cold absolute alcohol. This is repeated

TABLE I

CLOTTING PROPERTIES OF EXPERIMENTALLY PREPARED REAGENTS AT VARIOUS STAGES IN COURSE OF PREPARATION. 5° C. PROTOCOL

	CLOTTING TIME
1 Fresh dog blood	2 min
2 Twice centrifuged citrate plasma + Ca	4 min
3 Twice centrifuged citrate plasma + Ca + cephalin	3 min
4 Berkefeld (F) filtered plasma (2) + Ca	9 min
5 Berkefeld (F) filtered plasma (2) + Ca + cephalin	14 min
6 Mg(OH) <sub>2</sub> adsorbed plasma (1) + Ca	No clot
7 Mg(OH) <sub>2</sub> adsorbed plasma (4) + Ca + cephalin	45 min
8 Fibrinogen from (6) (first precipitation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ) + Ca	No clot
9 Fibrinogen from (6) (first precipitation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ) + Ca + cephalin	12 hrs
10 Fibrinogen from (8) (second precipitation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ) + Ca	No clot
11 Fibrinogen from (8) (second precipitation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ) + Ca + cephalin	Trace (18 hrs)
12 Fibrinogen from (10) (third precipitation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ) + Ca	No clot

15 10 5

19

ml

b.e. = benzene extracted<sup>8</sup>

three or four times until the ethereal solution remains perfectly clear after overnight cooling to 0° C, denoting freedom from cerebrosides. The final alcoholic deposit is a snowy white material, which has been kept under absolute alcohol for two years without undergoing oxidation or detectable loss in coagulative function. Cephalin solutions are prepared by evaporating 5 to 10 cc of the alcoholic suspension in a tared flask. An air jet has proved more convenient than the water bath. By adding the same number of cubic centimeters of distilled water as there are milligrams of dried cephalin, a convenient 1:1000 stock solution is obtained. Other dilutions may be made up as required. Aqueous cephalin solutions should be freshly prepared for all quantitative tests.

**Routine Control Tests**—1 One cubic centimeter of fibrinogen incubated with 0.25 cc each of 1:1000 cephalin and N/10 calcium chloride should give no trace of clot in twenty-four hours (38° C), indicating complete absence of prothrombin.

2. Each batch of *cephalin* solution should be tested with calcium salt and prothrombin-free fibrinogen. We have never observed any coagulant power attributable to the cephalin per se.

3. Fibrinogen should not be coagulated by any *prothrombin* preparation, with or without added cephalin, in the absence of added calcium salts.

The data in the accompanying protocol (Table I) show the modifications of the coagulation reactions at each stage in the experimental procedures.

*Thrombin Formation Tests.*—Various mixtures of prothrombin, cephalin, and calcium, together with any other agent (e.g., heparin) it is desired to test, are mixed and held at a chosen temperature (e.g., 20° C. v. infra), and 0.5 c.c. samples are removed at intervals. Each sample is added to 1 c.c. of prothrombin-free fibrinogen in a 10 mm. diameter Wassermann tube in a suitable rack, maintained at 38° C. (thermostatically-controlled water bath), and the clotting time (C.T.) noted. Clotting time plotted against the age of the thrombic mixture (timed from the addition of the calcium salt) gives the *activation curve* for the particular thrombic mixture under investigation. If the same prothrombin and fibrinogen preparations (known to be stable—v. infra) are used throughout, the character of the activation curve is a function of (a) the controlled physical conditions and (b) the amounts of the other reagents used.

#### DISCUSSION

It is immediately obvious from the data in the accompanying tables that *thrombic activity*, as indicated by clotting time, is not a fixed property of any given thrombic mixture, but rather a dynamic process which must be evaluated with reference to the sequence of change. Unless the particular point in the series of values can be specified on exact experimental grounds, no significance can attach to the use of clotting time as a measure of thrombic activity. It becomes necessary, therefore, to determine the conditions under which the activation curve may be regarded as standardized.

The first consideration relates to the purity and stability of the reagents used. The second factor is the set of physico-chemical conditions, including, for instance, pH, temperature, and dilution variables. The present communication represents a critical examination of these technical problems with a view to proceeding ultimately to an examination of the third variable, viz., quantitative differences relating to each of the specific factors involved.

*Purity of Reagents.*—The ionizable calcium salt<sup>7, 9, 22</sup> and the *cephalin* are chemically pure. Our phospholipid was free from traces of choline and analyzed, almost exactly, N:P::1:1, all the N being in NH<sub>2</sub> form. Its calcium compound<sup>27</sup> did not precipitate, but remained in colloidal solution, with the accompanying acid shift in pH readily demonstrated by bromthymol blue indicator.

*Prothrombin* (fibrinogen-free) is, unavoidably, a crude protein (or its degradation products), undoubtedly of plasma origin. In an experience including over fifty preparations we have on only one occasion (and that to a very slight

We are indebted to Dr. Quiek of Marquette University for a supply of his crude brain extract ("thromboplastin"<sup>21</sup>). On simple recalcification it clotted prothrombin-free fibrinogen in about seven minutes, showing inadequate purification from coagulant precursors.

extent) observed any "spontaneous" (i.e., unexplained) activation. It may be concluded that added calcium is essential for its conversion into thrombin. The anomalous data in the literature<sup>1, 15, 16, 20</sup> need searching investigation of this point. Owing to such "spontaneous" activation, doubt attaches to the claims of Howell and his pupils (op. cit.) that prothrombin may be converted into thrombin by such agents as alcohol, chloroform, etc., a finding we have been unable to confirm. Using one tenth volume of 0.005 per cent of Northrop and Kunitz's crystalline trypsin (kindly supplied by the Rockefeller workers) we have confirmed Eagle's<sup>4</sup> demonstration of coagulant activity in trypsinized prothrombin solutions (without added calcium or cephalin). There are, however, sufficient minor differences from the usual thrombin system to warrant doubt that the activation is the same phenomenon in both cases.

Calcium alone always develops a relatively weak coagulant. Cephalin enormously improves the thrombin activity and earlier data<sup>8, 21</sup> lead us to the conclusion that calcium alone is insufficient if sources of "available" phospholipid can be excluded. The Howell prothrombin preparation is usually "poor in available cephalin" and the practicability of working with our preparations even in quantitative studies on the cephalin factor is borne out by the fact that significant clotting time differences (at the minimal zone) accompany variations amounting to but 0.0005 mg. of added cephalin per 5 c.c. of prothrombin solution. It is not recommended to attempt further control of the cephalin factor by any routine use of lipid solvents.

*Fibrinogen*—On similar grounds to those discussed in connection with prothrombin, we may concede that fibrinogen is a plasma protein fraction free from "significant" calcium and containing only a small amount of "available" phospholipid.<sup>8</sup> It is prothrombin free.

*Antithrombin Factors*—The need for a considerable amount (of the order of one in several hundreds) of cephalin to neutralize minimal (one in several thousands) quantities of added heparin is recorded in another report.<sup>10</sup> Since one part of cephalin in several millions is quite active in our system, there can not possibly be any significant antithrombin factor content.

*Stability of Reagents*—The calcium chloride solutions require no comment. The keeping properties of cephalin solutions have been studied. A 1:1,000 solution, which remains clear and odorless, may be kept in the icebox for a week without demonstrable weakening of its clotting powers. Weaker solutions deteriorate more rapidly.

We have previously noted<sup>9</sup> the excellent stability of our prothrombin preparations but it is advisable to run controls before and after any extended series of quantitative tests, especially with diluted solutions. The fibrinogen solutions are highly satisfactory as a rule, but some denaturation may occur during the preparation despite every technical care. Close adherence to the cited method of preparation is recommended. Dialysis is unnecessary and undesirable.<sup>20</sup> Denaturation phenomena in relation to clotting have been considered by Fischer<sup>11, 12</sup> and Wohlisch.<sup>23</sup> A cool temperature is an important means of controlling denaturation of fibrinogen.

produced. That is why we always time our clots from the commencement. To wait for a solid clot in the presence of a weak thrombin introduces the consideration of amount of fibrin formed, and this, of course, is governed by a different set of factors, notable among which are questions related to quantity of thrombin and weight of the clot (Eagle<sup>2</sup>), neither of which can as yet be dignified with a biochemical significance.

*D. Temperature.*—The experiments of Table IV afford a good illustration of the well-known dependence of the rate of thrombin formation upon temperature. Attention is called, however, to the relationship of temperature to the rate of thrombin deterioration ("metathrombin"? formation). In the series A, the incubation of the thrombic mixture was continued at 38° C., with the evident result that the potency of the thrombin fell off quite rapidly after a brief optimal period. In experiment B, a portion of the identical mixture was removed after six minutes of incubation and immediately cooled and kept at 15° C. The stabilization of the thrombin by the lowering of the temperature is strikingly demonstrated.

TABLE IV

## EFFECT OF TEMPERATURE ON THROMBIN FORMATION AND STABILITY

Thrombic mixture = 10 c.c. prothrombin + 1 c.c. 1:1000 cephalin + 1 c.c. N/10 CaCl<sub>2</sub>  
T:F = 0.5 c.c. : 1.0 c.c.

Clotting times (seconds) at 38° C.

INCUBATION PERIOD =	1'	2'	5'	7'	10'	20'	30'	60'	90'	120'
A. 38° C. throughout	hrs.	hrs.	40"	40"	42"	85"	140"	1200"	hrs.	hrs.
B. 38° C. for 8 min. then at 15° C.*	hrs.	hrs.	40"	40"	41"	45"	45"	45"	50"	60"
C. 15° C. throughout	hrs.	hrs.	hrs.	---	210"	75"	30"	30"	30"	30"

\*Clotting tests of series B performed 1 min. earlier than times listed.

For "standard" conditions it is recommended that a temperature of 15° to 20° C. be employed for the thrombin activation, accepting the inconvenience of some delay in the process of activation, rather than the risk that differences in the activation curves be due to uncontrolled thrombin deterioration instead of to the variable which is being tested. The use of a temperature of 38° C. for the actual clotting test is justified by the speeding up of the clotting time so that there is seldom a long enough period for appreciable "metathrombin" formation. In certain delayed coagulations, however, this possibility may require further investigation.

Another illustration of the importance of temperature is offered in the data of Experiment V, which was devised in order to emphasize the necessity for controlling thrombin stability in evaluating relative thrombic activity by any dilution method.

*Experiment V.*—Thrombic mixture = 15 c.c. prothrombin + 1.5 c.c. 1:1,000 cephalin + 1.5 c.c. N/10 calcium chloride. After five, ten, thirty minutes' incubation, at 38° C., the clotting times of test samples were 30, 55, 210 seconds, respectively. Between the seventh and ninth minutes, dilutions of 1:2, 1:4, 1:8, and 1:16 were made and kept at 38° C. Between the eleventh and sixteenth minutes, these gave clotting times of 78, 145, 240, and 540 seconds, respectively. Between the thirtieth and thirty-fifth minutes, the corresponding tests yielded

clotting times of 175, 225, 360 and 570 seconds, a marked deterioration which would quite invalidate the use of these values as standards of reference for calculating "thrombin percentage" (Eagle *op cit*). Another series of the same dilutions was made up at approximately the same time as the first series. The second group, however, was kept at 15° C. The clotting times were (a) 72, 140, 220, 450 seconds respectively (ninth to eleventh minutes) and substantially the same, *viz.*, (b) 72, 140, 215, 460 seconds, respectively (thirtieth to thirty fifth minutes), some twenty minutes later.

It is concluded that the specific influence of thrombin concentration upon clotting time (*cf. supra*) makes it practicable to use a dilution method for converting thrombin activity (clotting time) into relative thrombin values (e.g., percentages), provided that suitable controls are made to assure stability of the thrombin throughout the experimental period. There is, however, no particular value in such additional procedures where the pertinent information can be read directly off the prothrombin activation curves.

### CONCLUSIONS

It is evident from the foregoing technical considerations that the standardization of procedures for the biochemical investigation of coagulation reactions can be achieved to a sufficiently satisfactory extent. The manipulations and controls are somewhat tedious and demand considerable care, but the results offer the possibility of a distinct reliance upon even small changes in clotting-time data in elucidating a variety of variables.

### SUMMARY

The standardization of coagulation techniques involves the preparation of isolated reagents (e.g., calcium salts, cephalin, prothrombin, and fibrinogen) controlled for "purity" and stability.

Physical variables necessitating investigation and control include pH, temperature, and dilution factors.

It is recommended that a pH slightly on the alkaline side (about 7.5) be maintained by controlling the test systems with phenol red indicator. A temperature not exceeding 15° to 20° C. is necessary to prevent thrombin deterioration. In most cases a temperature of 38° C. is preferred for the actual coagulation tests.

Under conditions of thrombin stability and controlled dilution variables, the clotting time values become reliable data of reference for the comparative investigation of a large number of experimental variations in the coagulation systems.

### REFERENCES

1. Cekada, E. B. The Preparation and Properties of Prothrombin, *Am. J. Physiol.* 78: 512, 1926.
2. Eagle, H. Studies on Blood Coagulation. I. The Role of Prothrombin and of Platelets in the Formation of Thrombin. II. The Formation of Fibrin From Thrombin and Fibrinogen, *J. Gen. Physiol.* 18: 531, 547, 1935.
3. Eagle, H., and Baumberger, J. P. Studies on Blood Coagulation. III. On the Constancy of Hydrogen Ion Concentration During Coagulation of Fibrinogen by Thrombin, *J. Gen. Physiol.* 18: 809, 1935.
4. Eagle, H., and Harris, T. N. Studies on Blood Coagulation. V. The Coagulation of Blood by Proteolytic Enzymes (Trypsin, Papain), *J. Gen. Physiol.* 20: 543, 1937.

5. Ferguson, J. H.: Cephalin Content of Prepared Fibrinogen and Prothrombin Solutions, *Proc. Soc. Exper. Biol. & Med.* 31: 929, 1934.
6. Ferguson, J. H., and DuBois, D.: Observations on the pH of Clotting and Citrated Blood, *J. LAB. & CLIN. MED.* 21: 663, 1936.
7. Ferguson, J. H.: The Blood Calcium and the Calcium Factor in Blood Coagulation, *Physiol. Rev.* 16: 640, 1936.
8. Ferguson, J. H.: An Experimental Analysis of Coagulant Activation, *Am. J. Physiol.* 117: 587, 1936.
9. Ferguson, J. H.: An Intermediary Calcium Complex in Blood Coagulation, *Am. J. Physiol.* 119: 755, 1937.
10. Ferguson, J. H.: Modus Operandi of Commercial Heparin, *Proc. Soc. Exper. Biol. & Med.* 37: 23, 1937.
11. Fischer, A.: Studies on Coagulation of the Blood, *Jap. J. Exper. Med.* 13: 223, 1935.
12. Fischer, A.: Über die Reaktionsmechanismus der Blutgerinnung, *Biochem. Ztschr.* 259: 67, 1933.
13. Fuchs, H. J., and Hartmann, E.: Ueber Proteolytische Fermente im Serum. XII. Die Bedeutung des Komplements bei der Blutgerinnung, *Ztschr. f. Immunitätsforsch. u. exper. Therap.* 58: 14, 1928.
14. Goddard, C. H.: The Effect of Filtration Through a Berkefeld Filter Upon the Coagulability of Oxalated Plasma, *Am. J. Physiol.* 35: 333, 1914.
15. Howell, W. M.: The Preparation and Properties of Thrombin, Together With Observations on Antithrombin and Prothrombin, *Am. J. Physiol.* 26: 453, 1910.
16. Howell, W. H.: Prothrombin, *Am. J. Physiol.* 35: 474, 1914.
17. Howell, W. H.: Theories of Blood Coagulation, *Physiol. Rev.* 15: 435, 1935.
18. Kugelmass, I. N.: Contribution Physico-Chimique au Mécanisme de la Coagulation du Sang, *Arch. internat. de physiol.* 21: 139, 1923.
19. Levene, P. A., and Rolf, I. P.: A Note on the Preparation of Cephalin, *J. Biol. Chem.* 74: 713, 1927.
20. Mellanby, J.: Prothrombase—Its Preparation and Properties, *Proc. Roy. Soc. S. B., London* 107: 271, 1930.
21. Mills, C. A.: Is Cephalin Necessary in the Activation of Prothrombin? *Chinese J. Physiol.* 1: 435, 1927.
22. Nordbö, R.: Über die Aktivitätskoeffizienten der Kalziumionen und der Oxalationen im Plasma und über die Bedeutung der Kalziumionenkonzentration für die Gerinnung des Blutes, *Skandinav. Arch. f. Physiol.* 75: Suppl. 11, 1936.
23. Quick, A. J.: On the Relationship Between Complement and Prothrombin, *J. Immunol.* 29: 87, 1935.
24. Quick, A. J.: On Various Properties of Thromboplastin (Aqueous Tissue Extracts), *Am. J. Physiol.* 114: 282, 1936.
25. Smith, H. P., Warner, E. D., and Brinkhous, K. M.: Lung Extract and Blood Clotting, *Am. J. Physiol.* 107: 63, 1934.
26. Thudichum, J. L. W.: A Treatise on the Chemical Constitution of Brain, 1884.
27. Wadsworth, A., Maltaner, F., and Maltaner, E.: Studies on the Activity of Cephalin as It Relates to the Coagulative and Complementary Properties of the Blood, *J. Immunol.* 30: 417, 1936.
28. Wöhlisch, E.: Die Physiologie und Pathologie der Blutgerinnung, *Ergebn. d. Physiol.* 28: 443, 1929.

## MULTIPLE PRIMARY MALIGNANT TUMORS\*

J D KIRSHBAUM, M D, M S, AND F L SHEVELA, JR, B S, B M,  
CHICAGO, ILL

**M**ULTIPLE primary malignant tumors in the same individual are of great interest since they demonstrate that a malignant neoplasm does not infer immunity against a second malignant tumor. The literature contains many reports describing multiple malignant tumors but in reviewing this literature one finds discrepancies as to the frequency of this combination as well as the criteria applied in determining whether two tumors are independent of each other.

Bilhott<sup>1</sup> is credited with having reported the first case of multiple primary tumors in 1869. Fried<sup>2</sup> considered the condition very rare, finding one case in 1,000 post mortem examinations. Muller,<sup>3</sup> in reviewing 5,012 autopsies over 20 years of age, noted 1,121 tumors, 18 or 1.6 per cent had more than one cancer. Harrington<sup>4</sup> studied 1,100 cases of carcinoma of the breast and found the second breast in 37 instances the site of carcinoma either simultaneously or subsequently. Thirteen cases he considered as multiple primary cancers, an incidence of 1.1 per cent. Hansemann<sup>5</sup> found 5 cases out of 1,000 tumor cases. Reddel,<sup>6</sup> quoted by Ewing,<sup>7</sup> reported 14 cases from a series of 1,225 cases. Hanlon, after carefully reviewing the literature, reported 48 cases. Puhz,<sup>8</sup> in a series of 6,718 necropsies from the second Budapest Pathological Institute, found 1,559 tumors, 219, or 14.04 per cent were multiple growths, of which 5, or 0.21 per cent, were multiple primary malignant neoplasms. Egh,<sup>9</sup> quoted by Murray, in reviewing 966 tumors at the Basel Pathological Institute, found a multiplicity of tumors in 263, or 27.3 per cent, of which there were 20 multiple malignant growths, or 2.07 per cent. Murray<sup>10</sup> reported a series of 4,219 examinations from the Oslo Cancer Institute to have had 32 multiple malignancies, or 1 per cent, of the cases. Harbitz<sup>11</sup> found 16 cases, or 3 per cent in 524 cancer autopsies. McNamara<sup>12</sup> found one case in 364 autopsies.

Single cases by Bosq,<sup>13</sup> Yamagiwa,<sup>14</sup> Luchsinger,<sup>15</sup> Ball and Reynolds,<sup>16</sup> Jolkwer,<sup>17</sup> Periotte,<sup>18</sup> Winter,<sup>19</sup> White,<sup>20</sup> Borgen and Rankin,<sup>21</sup> are described. Also two cases by New and Childrev,<sup>22</sup> Dusehl,<sup>23</sup> and Seebof.<sup>24</sup>

Pearson<sup>25</sup> reported similar tumors in both testes, and reviewed 46 primary bilateral tumors of the testes.

Hurt and Broders<sup>26</sup> found 71 cases of multiple primary malignant neoplasms, or 3.34 per cent, in 2,124 patients at the Mayo Clinic. The majority of these cases were from surgical specimens and not post mortem material.

\*From the Department of Pathology, Cook County Hospital. Dr. Walter Schiller, Director and the Department of Surgery, Northwestern University.  
Received for publication May 16 1938.



TABLE I  
SUMMARY OF 1411 CASES OF CARCINOMA

LOCATION OF TUMOR	NO. OF TUMORS AS PRIMARY CAUSE OF DEATH	NO. OF INCIDENTAL TUMORS
<i>Respiratory Tract</i>		
1. Oral cavity	33	3
2. Tonsil	7	0
3. Pharynx	14	1
4. Larynx	15	0
5. Lungs	144	0
<i>Gastrointestinal Tract</i>		
1. Esophagus	82	1
2. Stomach	255	18
3. Intestines		
a. Large	151	22
b. Small	8	0
4. Liver		
a. Primary	26	0
b. Bile ducts	13	2
5. Gall bladder	37	2
6. Extra hepatic bile ducts	49	1
<i>Male Sex Organs</i>		
1. Testicle	15	0
2. Prostate	68	22
3. Seminal vesicle	1	0
<i>Female Sex Organs</i>		
1. Urethra	1	0
2. Ovaries	34	1
3. Uterus		
a. Cervix	77	6
b. Corpus	14	1
4. Vagina and vulva	5	0
5. Breast	70	7
<i>Genitourinary Tract</i>		
1. Kidney	43	26
2. Ureters	0	1
3. Urinary bladder	23	2
<i>Endocrine Organs</i>		
1. Pancreas	67	0
2. Pituitary	2	0
3. Thyroid	13	0
4. Adrenal	2	0
5. Thymus	1	0
<i>Miscellaneous</i>		
1. Skin	18	7
Total	1288	123

Owen<sup>27</sup> found 4.7 per cent of 3,000 cases of primary multiple malignant tumors at the Barnard Free Skin and Cancer Hospital.

Warren and Gates<sup>41</sup> made an extensive review of the literature on the subject of multiple primary malignancies up to the year 1932. They found an incidence of 1.84 per cent of multiple primary malignancies in their collected series of 794 cases of carcinoma. In their own series of 1,078 autopsies in which carcinoma was the primary cause of death, 40 cases were encountered in which there were multiple primary carcinomas giving an incidence of 3.17 per cent.

Burke<sup>42</sup> from 1924 to 1935 reported 583 cancers with an incidence of 28.6 per cent in a series of 2,033 autopsies. In this series there were 46 cases of multiple primary carcinoma, with an incidence of 7.8 per cent.

TABLE II  
SUMMARY OF 25 CASES\*

CASE NO	AGE	SEX	RACE	ORGAN INVOLVED			METASTASES	
				TUMOR NO 1	TUMOR NO 2	TUMOR NO 3	TUMOR NO 1	TUMOR NO 2
1	65	M	C	Stomach	Sigmoid	----	Present	Absent
2	66	M	C	Stomach	Sigmoid	----	Present	Present
3	50	M	W	Stomach	Esophagus	----	Present	Absent
4	81	M	W	Stomach	Prostate	----	Present	Absent
5	75	M	W	Transverse colon	Skin	----	Absent	Absent
6	74	F	W	Sigmoid	Rectum	----	Present	Absent
7	77	M	W	Sigmoid	Rectum	----	Absent	Absent
8	76	M	W	Rectosigmoid	Kidney	----	Absent	Present
9	66	M	W	Colon	Tonsil	----	Present	Present
10	77	M	W	Common duct	Hepatic flexure of colon	----	Present	Absent
11	56	M	W	Common duct	Stomach	----	Present	Absent
12	54	F	W	Hepatic duct	Rectum	----	Present	Absent
13	69	F	W	Gall bladder	Rectum	----	Present	Absent
14	57	M	C	Liver	Rectum	----	Absent	Absent
15	53	M	W	Pancreas	Stomach	----	Present	Absent
16	60	F	C	Lung	Uterus	----	Present	Absent
17	68	M	W	Lung	Prostate	----	Present	Absent
18	59	F	W	Lung	Cervix	----	Present	Absent
19	69	M	W	Lip	Liver	Prostate	Present	Absent
20	47	F	W	Cervix	Breast	----	Present	Present
21	65	M	W	Kidney	Rectum	----	Present	Absent
22	35	F	W	Adrenal	Colon	----	Present	Present
23	70	M	W	Esophagus	Kidney	----	Absent	Absent
24	52	M	W	Pyramidal sinus and aryepiglottic fold	Stomach	----	Absent	Absent
25	60	F	W	Thyroid	Stomach	----	Present	Absent

\*Average age—63.3 years, females—white 7 colored 1 males—white 14, colored 3

The material studied in our paper was selected from 10,870 consecutive autopsies performed at the Cook County Hospital from 1929 to May, 1938, inclusive. There were 1,411, or 12.98 per cent, carcinomas encountered. One thousand two hundred and eighty-eight of the carcinomas were the primary cause of death, while 123 of the carcinomas were an incidental finding at necropsy. Sarcomas and malignant neurogenic tumors were excluded in our group of cases (see Table I). Twenty-five cases presented multiple primary malignant neoplasms, or 1.77 per cent, of the malignant neoplasms. The 25 cases are briefly summarized in Table II.

#### CASE REPORTS

CASE 1.—A colored male, aged 65 years. *Clinical Diagnosis* Carcinoma of the stomach. *Anatomic Diagnosis* (1) Ulcerated, disk-shaped adenocarcinoma of the lesser curvature of the stomach, (2) polyp of the sigmoid colon with malignant transformation. *Metastases*: (1) to the perigastric, peripancreatic, periaortic lymph nodes, liver, perirenal fat tissue, and mucosa of the small intestine.

CASE 2.—A colored male, aged 66 years. *Clinical Diagnosis* (1) Atrophic cirrhosis, (2) carcinoma of the stomach. *Anatomic Diagnosis* (1) Ulcerated medullary carcinoma of the fundus of the stomach and perforation into the retrogastric space and formation of a retrogastric abscess, (2) polyp of sigmoid colon with malignant transformation. *Metastases* (1) to the perigastric, peripancreatic, and mesenteric lymph nodes, (2) to the liver.

CASE 3.—A white male, aged 50 years. *Clinical Diagnosis* (1) Carcinoma of the esophagus or upper cardia with metastases to the vertebrae and cervical lymph nodes; (2)

myocardial degeneration. *Anatomic Diagnosis:* (1) Adenocarcinoma of the cardiac end of the stomach with stenosis of the cardiac orifice; (2) hornifying, anaplastic, squamous-cell carcinoma of the esophagus. *Metastases:* (1) to the pleura, liver, greater omentum, periaortic lymph nodes, peritoneum, and diaphragm.

CASE 4.—A white male, aged 81 years. *Clinical Diagnosis:* Carcinoma of the stomach with pyloric obstruction. *Anatomic Diagnosis:* (1) Ulcerated mucus-producing adenocarcinoma of the prepyloric portion of the stomach; (2) adenocarcinoma of the prostate gland. *Metastases:* (1) to the perigastric, mesenteric, peribiliary, and peripancreatic lymph nodes, and to the peritoneum.

CASE 5.—A white male, aged 75 years. *Clinical Diagnosis:* Syphilitic heart disease. *Anatomic Diagnosis:* (1) Adenocarcinoma of the transverse colon with moderate stenosis of the lumen; (2) a small basilioma of the nose. *Metastases:* None.

CASE 6.—A white female, aged 74 years. *Clinical Diagnosis:* Acute intestinal obstruction due to a carcinoma. *Anatomic Diagnosis:* (1) Annular constricting adenocarcinoma of the sigmoid colon with partial obstruction of the lumen; (2) plaque-like adenocarcinoma of the rectum. *Metastases:* to the liver.

CASE 7.—A white male, aged 77 years. *Clinical Diagnosis:* Carcinoma of the sigmoid causing partial obstruction. *Anatomic Diagnosis:* (1) Stenosing carcinoma at the junction of the descending and sigmoid colon; (2) papilloma of the rectum with carcinomatous transformation. *Metastases:* None.

CASE 8.—A white male, aged 76 years. *Clinical Diagnosis:* (1) Carcinoma of the recto-sigmoid junction with metastases to the liver; (2) generalized arteriosclerosis; (3) myocardial sclerosis with mild decompensation. *Anatomic Diagnosis:* (1) Ulcerating, fungating, adenocarcinoma of the rectosigmoid junction; (2) hypernephroid carcinoma of the upper pole of the left kidney. *Metastases:* (1) None to the liver; (2) to the kidney capsule.

CASE 9.—A white male, aged 66 years. *Clinical Diagnosis:* Biopsy of tonsil and palate revealed a squamous-cell carcinoma. *Anatomic Diagnosis:* (1) Colloid carcinoma of the sigmoid colon; (2) hornifying epidermoid carcinoma of the left tonsil. *Metastases:* (1) to the liver, sternum, thyroid, and the left adrenal; (2) extension into the soft palate.

CASE 10.—A white male, aged 77 years. *Clinical Diagnosis:* (1) Carcinoma of the intestine with metastases to the liver, or (2) carcinoma of the pancreas. *Anatomic Diagnosis:* (1) Adenocarcinoma of the common duct at the junction with the cystic duct; (2) circular, fungating, mucous carcinoma of the hepatic flexure of the colon. *Metastases:* (1) to the hepatic and biliary lymph nodes, gall bladder, and the liver.

CASE 11.—A white male, aged 56 years. *Clinical Diagnosis:* (1) Carcinoma of the bile ducts or pancreas; (2) Catarrhal jaundice; (3) silent stone in the common duct. *Anatomic Diagnosis:* (1) Adenocarcinoma of the common duct at the junction with the cystic duct occluding the opening of the cystic duct; (2) stenosing, disk-shaped adenocarcinoma of the pyloric portion of the stomach. *Metastases:* (1) to the peribiliary lymph nodes, liver, and the wall of the stomach.

CASE 12.—A white female, aged 54 years. *Clinical Diagnosis:* (1) Carcinoma of the head of the pancreas, or (2) carcinoma of the bile ducts with obstruction. *Anatomic Diagnosis:* (1) Mucus-producing adenocarcinoma of the hepatic ducts completely obliterating the lumen; (2) ulcerating carcinoma of the rectum. *Metastases:* (1) to the liver and the left lower lobe of the lung.

CASE 13.—A white female, aged 69 years. *Clinical Diagnosis:* Gastrointestinal malignancy. *Anatomic Diagnosis:* (1) Adenocarcinoma of the gall bladder; (2) multiple (2) polyps of the rectum with malignant transformation of one of them. *Metastases:* (1) to the liver and peribiliary lymph nodes.

CASE 14—A colored male, aged 57 years *Clinical Diagnosis* (1) Syphilitic aortic insufficiency, (2) carcinoma of the liver, probably secondary to the stomach with ascites *Anatomic Diagnosis* (1) Carcinoma of the liver, (2) polyp of the transverse colon with carcinomatous transformation *Metastases* (1) with tumor invasion and obstruction of the portal vein and secondary thrombosis extending into the superior mesenteric vein

CASE 15—A white male, aged 53 years *Clinical Diagnosis* Recurrent carcinoma of the stomach with metastases to the portal lymph nodes and compression of the bile ducts *Anatomic Diagnosis* (1) Adenocarcinoma of the head of the pancreas with compression of the common bile duct, (2) multiple (2) polyps of the stomach with early malignant transformation of one of them *Metastases* (1) to the liver, peripancreatic lymph nodes, and the periaortic lymph nodes, tumor thrombosis of the hepatic and splenic arteries, and to the wall of the gall bladder

CASE 16—A white female, aged 60 years *Clinical Diagnosis* (1) Carcinoma of the corpus uteri with metastases to the left lung or (2) carcinoma of the right ovary and a secondary bronchiogenic carcinoma, (3) arterio sclerotic heart disease with mitricular fibrillation *Anatomic Diagnosis* (1) Medullary adenocarcinoma of the main bronchus of the left upper pulmonary lobe, (2) fungating adenocarcinoma of the corpus uteri with extension into the cervical canal and a suppurative endocervicitis *Metastases* (1) to the left upper pulmonary lobe and to the left pulmonary hilar lymph nodes

CASE 17—A white male, aged 58 years *Clinical Diagnosis* (1) Malignancy of the lung, (2) bone tumor of the right radius *Anatomic Diagnosis* (1) Very anaplastic, undifferentiated bronchiogenic squamous cell carcinoma of the bronchus of the right upper lobe, (2) adenocarcinoma of the prostate *Metastases* (1) to all the pulmonary lobes, vertebral body, ribs, adrenals, kidneys, myocardium, stomach, and to the skin of the scarp

CASE 18—A white female aged 59 years *Clinical Diagnosis* (1) Multiple metastases to lungs, mediastinum, and subcutaneous tissue of the chest either a carcinoma of the cervix or a sarcoma of the right orbit *Anatomic Diagnosis* (1) Medullary, round cell carcinoma of the middle lobe of the right lung, (2) ulceration and fibrosis of the cervix uteri with obliteration of the external os (radium sterilized carcinoma of the cervix), (3) sarcoma of the right orbit *Metastases* (1) to both lungs pulmonary hilar peritracheal, posterior mediastinal, peripancreatic, periaortic lymph nodes, right serratus musculo and diaphragm, greater omentum, anterior mediastinum, both adrenals, both kidneys and under the capsule of the liver

CASE 19—A white male, aged 69 years *Clinical Diagnosis* (1) Carcinoma of the lip which was diathermized *Anatomic Diagnosis* (1) Epidermoid carcinoma of the upper lip, (2) periportal cirrhosis of the liver and early hepatocellular carcinoma of the right lobe, (3) adenocarcinoma of the prostate *Metastases* (1) to the submaxillary lymph nodes of both sides

CASE 20—A white female, aged 47 years *Clinical Diagnosis* A far advanced carcinoma of the right breast with multiple metastases *Anatomic Diagnosis* (1) Ulcerating hornifying, squamous cell carcinoma of the cervix, (2) scirrhous ductal carcinoma of the breast *Metastases* (1) to the body of the uterus vaginal wall rectum urinary bladder periaortic and perihilar lymph nodes and to the liver (2) to the skin right axillary lymph nodes, left clavicle, pleura, lung, and to the right adrenal

CASE 21—A white male, aged 65 years *Clinical Diagnosis* Annular constricting adenocarcinoma of the rectum with metastases to the lungs *Anatomic Diagnosis* (1) Malignant hypernephroid carcinoma of the left kidney (2) adenocarcinoma of the rectum *Metastases* (1) to the renal vein and pelvis, lungs, and liver

CASE 22—A white female, aged 75 years *Clinical Diagnosis* (1) A pseudomucinous cystadenoma of the left ovary with malignant transformation, or (2) a malignancy of the

colon. *Anatomic Diagnosis:* (1) Hypernephroid carcinoma of the left adrenal; (2) adenocarcinoma of the colon. *Metastases:* (1) invading the veins of the kidney and the inferior vena cava; metastases to the liver and to the right ovary; (2) to the liver and mesentery.

CASE 23.—A white male, aged 70 years. *Clinical Diagnosis:* Malignant hypernephroma. *Anatomic Diagnosis:* (1) Hornifying squamous-cell carcinoma of the upper one-third of the esophagus with marked constriction; (2) recurrent papillary adenocarcinoma of the right kidney. *Metastases:* None.

CASE 24.—A white male, aged 52 years. *Clinical Diagnosis:* Advanced pulmonary tuberculosis and tuberculous laryngitis. *Anatomic Diagnosis:* (1) Ulcerated squamous-cell carcinoma of the left pyriform sinus involving the aryepiglottic fold; (2) adenocarcinoma of the lesser curvature of the stomach.

CASE 25.—A white female, aged 60 years. *Clinical Diagnosis:* (1) From an x-ray of the chest and skull, an osteolytic malignant tumor of the parietal bone with metastasis to the lungs was considered. (2) The diagnosis remained uncertain as to the origin of the tumor. *Anatomic Diagnosis:* (1) Adenocarcinoma of the thyroid; (2) adenomyomatosis of the stomach with carcinomatous transformation. *Metastases:* (1) to both lungs, the right parietal bone, sacral bone, and the right peritracheal lymph nodes.

#### DISCUSSION

The 25 cases, with apparently two independent primary malignant tumors, were selected from a series of 1,411 malignant tumors. The tumors were situated in different organs, usually in different systems, and frequently showed different histologic structures. These criteria are necessary in order to exclude metastases that may simulate primary tumors. Billroth formulated three rules which he thought had to be adhered to in making a diagnosis of multiple primary malignant tumors: (1) each tumor must have a different histologic appearance, (2) the tumors must have different sites of location, and (3) each tumor must produce its own metastases. The above rules may apply to some cases, but they cannot be fulfilled in all cases of multiple primary malignant tumors. Tumors need not necessarily have a different histologic structure in order to be considered independent. This is illustrated by Case 4, which showed an adenocarcinoma of the stomach and an adenocarcinoma of the prostate. A single organ may be the site of two different primary malignant tumors (Jaffé<sup>28</sup> and Renner<sup>29</sup>), either from an embryologic basis or due to irritants. No metastasis may exist from either of the two tumors, i.e., as shown by 5 of the cases.

Goetze<sup>30</sup> offered the following criteria for the diagnosis of multiple primary malignant neoplasms: (1) the tumors must have the macroscopic and microscopic appearance of the usual tumors of the organs involved, (2) the exclusion of metastasis must be certain, and (3) the diagnosis may be confirmed by the character of the individual metastases. The third point was illustrated in only Case 22 of the series; the liver contained metastatic nodes of two distinct different histologic structures.

Wooley,<sup>31</sup> in an attempt to classify multiple primary malignant tumors, listed them into the following groups: (1) multiple tumors may affect the same tissue and produce identical tumors, or growths of different types; (2) affecting both of a pair of organs need not necessarily be of the same histologic type, e.g., both breasts; (3) affecting different regions of the same system need not

necessarily be of the same histologic type i.e. the genital tract, (4) different systems may be affected by different types of new growths

It is apparent that there is no standard definition to characterize multiple primary malignant tumors. One must keep in mind that metastases sometimes show considerable differences from the primary tumor. In the metastases metaplastic changes may be present that are not observed in the primary tumor.

Various theories have been offered for the pathogenesis of multiple primary malignant neoplasms. Ewing<sup>31</sup> states that "The rather common occurrence of two or more tumors in different or same organs of the same subject suggests nothing more than the coincidence in several organs of the general biological factors in the genesis of tumors." He suggests that when minute search is carefully conducted and all forms of tumor growths included the proportion of multiple primary tumors will be much greater. That there may be an endogenous cause, i.e., the abnormal distribution of chromosomes in nuclei has been often emphasized. Anomalies in cell division have been repeatedly observed.<sup>31-34</sup> There may be a mechanical developmental anomaly<sup>35</sup> or multiple foci of irritation. Toxins may persistently act in the same individual in two different locations.<sup>36</sup> Hadser<sup>3</sup> suggested that there may be multiple foci of embryonal displaced or superfluous cells as the cause. It was thought that in individuals with one tumor may exert an immunity against a second tumor. This belief was based on the experimental work of Ehrlich and Apolant<sup>36</sup> who showed that a transplanted malignant growth in mice conferred immunity against a second inoculation of the same or another tumor. Later workers have shown that this immunity diminishes after a short period. An individual with a malignant growth usually succumbs to it before a second malignant neoplasm develops.

The complex influence of an existing tumor upon neighboring cell groups may predispose to tumor growth.<sup>37</sup> Similar tumors in paired organs may exert different influences in each case. There may be widespread multiple foci of origin which may exhibit neoplastic growth one after the other, thus simulating the lateral diffusion of the tumor process.<sup>38</sup> As an explanation for the involvement of paired organs, Wooley<sup>32</sup> believed that the cells must be in a special state, so that whatever acted upon one organ whether the active proliferation was initiated by one factor or another, affected similarly its pair. Orr<sup>40</sup> was of the opinion that if accurate observations were recorded of the occurrence of multiple primary malignant tumors, the disputed question of whether one malignant tumor exercises an inhibiting effect on the development of a second would be settled.

The 25 cases described are briefly summarized in Table II. There were 17 males and 8 females. Twenty one of the cases were white and 4 were colored. The ratio of males to females in our series was 2.2:1, while in Burke's<sup>41</sup> series the ratio was 2:1. There were 7 cases in which the primary tumor showed no metastases, 19 showed metastases. Twenty one cases of the incidental tumors found at autopsy showed no metastases, 5 showed metastases. Two of the patients were under 50 years of age, 7 were between 50 and 59 years, 9 were between 60 and 69 years, and 7 were over 70 years, an average of 63.3 years.

The youngest male was 50 years, the oldest was 81 years, making an average of 66.0 years of age. The youngest female was 35 years, the oldest was 74 years, making an average of 57.2 years of age. In Hanton's<sup>7</sup> series, the average age was 62.6; Owen's<sup>27</sup> cases, 62.1 years; and Hurt and Broders'<sup>26</sup> as low as 50.4 years. The low age incidence given by Hurt and Broders, as compared to the authors' and other reports, may be because the material they studied was chiefly surgical specimens. The diagnosis of malignant tumors removed at operation is usually made earlier than the tumors found in autopsic material.

It is frequently noted that neoplasms of older people are less malignant than those of younger persons, and it appears obvious that the one with the slower growing tumor will live longer and thereby perhaps have a better chance of developing a second tumor. The above explanation may account for the frequent occurrence of multiple neoplasms in older people.

If one can generalize from a single case example, it may be said that the first malignant tumor does not influence or predispose to the formation of a second one, as exemplified in Case 8. In this case the first tumor was removed by radical operation, and four years later the patient succumbed to a second primary malignant tumor. There were no evidences of any recurrence of the first tumor.

The frequency with which multiple primary malignant tumors are encountered in the literature speaks against the possibility of one tumor conferring an immunity toward the development of a second independent neoplasm.

The colon was involved in 14 cases, the stomach in 8, and the kidney in 3. In 21 cases the gastrointestinal tract was the site of one of the two malignant tumors. The kidney and colon were the most frequent combination of organs to be affected.

The diagnosis of multiple primary malignant tumors was considered clinically in only one case. The second tumor was always, with the exception of the aforementioned case, an incidental finding. In many of the cases the symptoms produced by the multiplicity of tumors were confusing and in each case the findings were fitted together so as to conform with the diagnosis of a single malignant tumor. Frequently the confusion of a group of unrelated symptoms will be clarified if one will keep in mind the possibility that a patient may be afflicted with two or more independent unrelated conditions, whether it be two primary malignant tumors or two infectious diseases.

Only 4 of the cases presented metastases from each of the two tumors. This illustrates the dictum that in multiple primary malignancies each tumor need not necessarily produce its own type of metastasis in order to make a diagnosis of multiple primary neoplasms in the same individual. So often one of the tumors is slow growing, whereas the second produces extensive metastases and death. In 6 of the cases, the tumors produced no metastases. Only one of the 25 cases presented identical histologic changes in both primary tumors. The corollary that the microscopic appearance of the tumor usually differs when two different organs are affected is of diagnostic aid and for the most part an essential criterion, but is not always applicable to all cases. In 20 of the cases

here described, organs of two different systems were the sites of primary malignant tumor formation, while in two of them, three different organs were the sites of malignancy.

It is very apparent that there are no uniform steadfast laws that may be laid down to designate the diagnosis of multiple primary malignant tumors in the same individual in all given cases.

#### CONCLUSION

Twenty five cases of multiple primary malignant tumors selected from 1,411 malignant tumors are described, an incidence of 1.77 per cent.

The incidence of multiple tumors in surgical specimens is much higher and is given between 3.34 per cent and 4.7 per cent.

The colon was the site of one of the multiple tumors in 13 of the cases, or 52 per cent, the colon and kidney were the most frequent combination involved.

The average age was 63.3 years. Seventeen were males and 8 were females.

Multiple malignant neoplasms as an incidental finding without any immunity or direct relationship of one tumor for another in the same individual, are discussed.

Some individuals may be endowed with a congenital, or acquired, predisposition toward tumor formation. These factors may be considered as explanation for the presence of multiple primary malignant tumors in the same individual.

#### REFERENCES

- 1 Billroth, T. Die Allgemeine Chirurgie Pathologie u. Therapie, Berlin Reimer, 1889, p. 908.
- 2 Fried, D. M. Primary Double Cancer, Arch. Path. 5: 565, 1928.
- 3 Muller, R. F. Über multiple nichtsystematisierte Primäre Carcinome und ihre Häufigkeit, Ztschr. f. Krebsforsch. 31: 339, 1930.
- 4 Harrington, S. W. Carcinoma of the Breast, J. Lancet 50: 1, 1930.
- 5 Haesemann, V. Ztschr. f. Krebsforsch. 1: 183, 1904.
- 6 Redlich. Quoted by Ewing.
- 7 Hanlon, F. R. Multiple Primary Carcinomas, Am. J. Cancer 15: 2001, 1921.
- 8 Fuhr, L. Über die Multiplicität der Geschwülste, Ztschr. f. Krebsforsch. 24: 38, 1926.
- 9 Egli. Quoted by Murray.
- 10 Murray, J. A. Lunace Lecture on Multiple New Growths, Lancet 2: 800, 1927.
- 11 Harbitz, F. Quoted by McNamara.
- 12 McNamara, W. L. Multiple Tumors, J. Lab. & Clin. Med. 19: 219, 1933.
- 13 Bossi, P. Multiple Primary Tumors, Seminars Med. 1: 1748, 1932.
- 14 Yamaguchi, K., Tsukihira, S., and Morimoto, S. Experimentelle Studie über die Antikörperbildung gegen das Impfkarcinom bei Maus. Gann 21: 1, 1927.
- 15 Luchsinger, R. Zur Kasuistik der primären multiplen malignen Tumoren, Frankfurt Ztschr. f. Path. 40: 417, 1930.
- 16 Ball, R. P., and Reynolds, J. A. Multiple Primary Malignancies, M. J. & Rec. 126: 287, 1927.
- 17 Jolkwer, W. E. Über Gleichzeitiges Vorkommen multipler Geschwülste differenten Art, Arch. f. klin. Chir. 155: 142, 1929.
- 18 Perrotte, G. Multiple Tumors, Morgagni 70: 1889, 1923.
- 19 Winter, H. Multiple Primäre Blastombildung, Ztschr. f. klin. Med. 122: 627, 1932.
- 20 White, G. S. Double Primary Malignant Tumors of the Colon, Ann. Surg. 98: 186, 1933.
- 21 Bargen, J. A., and Ranlin, F. W. Multiple Carcinomata of the Large Intestine, Ann. Surg. 91: 583, 1930.
- 22 New, G. B., and Childrey, J. G. Tumors of the Tonsil and Pharynx, 357 Cases, Arch. Otolaryng. 14: 699, 1931.



23. Duschl, L.: Über Primäre Multiplizität von Geschwülsten, Deutsche Ztschr. f. Chir. 193: 77, 1925.
24. Seecof, D. P.: Multiple Primary Malignant Neoplasms, J. Cancer Research 8: 213, 1924.
25. Peirson, E. L., Jr.: Bilateral Tumors of the Testicle, J. Urol. 28: 353, 1932.
26. Hurt, H. H., and Broders, A. C.: Multiple Primary Malignant Neoplasms, J. LAB. & CLIN. MED. 18: 765, 1933.
27. Owen, L. J.: Multiple Malignant Neoplasms, J. A. M. A. 76: 1329, 1921.
28. Jaffé, R. H.: Sarcoma and Carcinoma of the Liver Following Cirrhosis, Arch. Int. Med. 33: 330, 1924.
29. Renner, M. J.: Primary Malignant Tumors of the Ureter, Surg., Gynec. & Obst. 52: 793, 1931.
30. Goetze, O.: Bemerkungen über Multiplizität primärer Carcinome, Ztschr. f. Krebsforsch. 13: 281, 1913.
31. Wooley, P. G.: Notes on Multiple Primary Tumors, Boston M. & S. J. 148: 1, 1903.
32. Ewing, J.: Neoplastic Disease, ed. 3, Philadelphia, 1928, W. B. Saunders Co., p. 36.
33. Boveri, T.: Zur Frage der Entstehung Maligner Tumoren, Jena, 1914.
34. Preisz, H.: Ueber die Entstehung der Neubildungen im Anschluss an eine Analogie, Ztschr. f. ärztl. Fortbild. 19: 174, 1922.
35. Albrecht, H.: Über das Karzinosarkom des Uterus, Frankfurt. Ztschr. f. Path. 2: 191, 1908.
36. Winiwarter, O.: Beitr. z. Statistik d. Karzinome, Stuttgart, 1878.
37. Hauser, D. A.: Quoted by Ewing.
38. Ehrlich, P., and Apolaut, H.: Beobachtungen über Maligne Mausetumoren, Berl. klin. Wchnschr. 72: 871, 1905.
39. Ribbert, H.: Das Carcinom des Menschen, Bonn, 1911, p. 367.
40. Orr, J. W.: Multiple Malignant Neoplasms, J. Path. & Bact. 33: 283, 1930.
41. Warren, S., and Gates, O.: Multiple Primary Malignant Tumors, Am. J. Cancer 16: 1358, 1932.
42. Burke, M.: Multiple Primary Cancers, Am. J. Cancer 27: 316, 1936.

# LABORATORY METHODS

---

## THE QUANTITATIVE UNRELIABILITY OF THE NITROPRUSSIDE TEST FOR SH AND SS\*

---

FREDERICK S. HAMMETT, PH D., AND SIDNEY S. CHAPMAN, B S,  
NORTH TRURO MASS

---

THE nitroprusside reaction is widely used for the colorimetric demonstration and estimation of sulfhydryl (SH) and disulfide (SS) in tissues and the products of their activities

The test as usually applied consists of first saturating an appropriate quantity of substance or solution with crystalline ammonium sulfate, then making it alkaline with one or two drops of concentrated 27-29 per cent ammonium hydroxide, and finally adding one or two drops of 5 per cent sodium nitroprusside solution. In the presence of SH a fleeting pink to purplish red color develops.

The color is not given by SS. This must first be reduced to SH. This is done by exposing the material to two or three drops of 5 per cent potassium cyanide or sodium cyanide for ten minutes, after which the above described procedure is employed.

Clearly such directions admit of many variations. It is but natural, therefore, that the test has not been quantitatively satisfactory. Although the compounds which give a like color and those which interfere with color development are very well known,<sup>1,2</sup> what effects, if any, the reagents themselves may have on variations in color change and intensity have yet to be established.

Obviously no reliable quantitative method can be devised until these are known. Nor can surety be had even of the relatively simple qualitative reaction. For although a positive reaction can fairly safely be taken as evidence of the presence of the sought-for groups, a negative reaction at borderline concentrations might conceivably be as much due to inhibition from the improper use of some reagent as to the absence of reacting sulfur compounds.

These principles make desirable an inquiry into the effects of the reagents of the nitroprusside test on its reaction with SH and SS. Such is here reported.

As test material we used from 0.5 cc to 1 cc of approximately M/10,000 cysteine, cystine, glutathione, and p-thiocresol. These were especially purified for us by the Chemical Division of the Lankenau Hospital Research Institute.

---

\*From the Marine Experimental Station of the Lankenau Hospital Research Institute, North Truro, Mass.

Received for publication January 21, 1933

Standard procedure was to keep all reagents except one constant and then observe the changes in intensity and duration of color with changes of the variant in order of use and concentration. The results are as follows:

*Sodium Nitroprusside:* This is the chromogenic compound. As such it is incomplete. Without alkalization it produces no sure color. The chromogenicity resides in some chemical grouping produced by the action of hydroxyl ion. The nature of this is as yet unknown.

The nitroprusside is usually used in 5 per cent water solution. This has a high color. When the  $-SH$  concentration is low (circa  $M/100,000$  to  $M/200,000$ ) so that the pink color is faint, the tint is obscured and even obliterated by the tint of the reagent. We have found a 1 per cent solution does away with this difficulty. It also is adequate for full color development with  $-SH$ .

The nitroprusside solution turns green in warm bright sunlight, be this direct or indirect. This, of course, masks the reaction with  $-SH$ . The addition of 0.1 c.c. concentrated ammonium hydroxide to each 10 c.c. prevents the decomposition. Although the addition of ammonium hydroxide is advocated by Giroud and Bulliard<sup>4</sup> as routine, we prefer plain water solution because ammoniated nitroprusside changes color on standing. This is evidence of undesired change in chemical character of the reagent.

It is emphasized that only freshly prepared nitroprusside solutions should be used. New solutions should be made up at least every hour when the assay is protracted.

*Ammonium Hydroxide:* Although ammonium hydroxide is the customary alkaline reagent, it seemed worth while to determine if the effect is attributable to  $OH$  ion and if  $NH_4$  ion participates.

To this end, from 0.10 c.c. to 0.25 c.c. of  $M/10$  sodium hydroxide,  $M/10$  sodium cyanide,  $M/50$  lithium carbonate, and  $M/15$  disodium phosphate were severally substituted for the concentrated ammonium hydroxide. Color was produced in all cases. This shows that  $OH$  ion makes sodium nitroprusside chromogenic for  $-SH$ .

In running the trials it was found that the color character differed with the cation. Thus while  $NH_4$  ions gave their characteristic pink,  $Na$  ions gave a reddish purple, and  $Li$  ions a distinct trend to orange and yellow. It is clear that the  $NH_4$  ion as such is a factor in the determination of the kind of color produced in the test.

The fact that other cations give other tints is important in any attempted quantitative use of the test. An unadjustable mixture of cations in the tissue or solution being tested could give colors which are unmatchable with that produced by ammonium hydroxide with a standard.

Studies were made of increasing ammonium hydroxide concentration to see if color intensity is related thereto. It was found that as ammonium hydroxide increased from 1.6 to 4.0  $M$  the color also increased; but that from 8.0  $M$  on, the color tended to fade out. Similar changes occurred in similar trials with the other  $OH$  ion sources. Clearly there is an optimum concentration for maximum color production. While this can be determined for pure solutions, its estab-

ishment in tissues and their extracts is unsure. On this basis alone the colorimetric estimation of SH in biologic material in terms of a standard solution is at best but an approximation.

Sodium phosphate solutions, particularly those which have stood for some time, act to inhibit color development, even though the pH is that to be expected for the M/15 concentration (viz., 8.04). This suggestion of phosphate interference has been borne out in work with root tip extracts. Here it was found that extracts buffered with 0.1 cc phosphate mixture to 1.5 cc solution gave much less color than unbuffered controls. It was not the buffering per se which was responsible, since citrate buffered extracts showed no such inhibition. It is evident that accurate quantitative estimation of SH by nitroprusside in the presence of any considerable amount of phosphates is impossible. Phosphate buffers should be avoided in any work where SH is to be determined by nitroprusside.

The chromogenic property of Na nitroprusside with SH is dependent upon OH ion action. The most controllable source seems to be ammonium hydroxide, probably because of its low  $pK$  (1.8 by 10). This is important since development of color intensity is affected by the amount of OH ion present.

*Ammonium Sulfate* When this salt is added to the nitroprusside, ammonium hydroxide, -SH mixture the color is intensified. The intensification increases with increase in salt concentration up to saturation. Control of this factor rests in being sure that saturation is attained. The practice of adding saturated solutions is inadequate. The solid salt must be used—not its solution—and there must be an excess thereof. Since the white of the crystals acts to dilute the pink of the positive test, balance must be had between saturation and excess salt. This can be determined in each case. Considerable latitude is allowable. Ammonium sulfate, therefore, of all the reagents used in the nitroprusside test is the one about which the least anxiety need be felt as a disturbing factor in semiquantitative assays.

The color produced fades rapidly. It would seem logical in attempting quantitative work to add the intensifying reagent last rather than first in order that its effect may not be diminished by the interval between beginning and ending of reagent introduction. This has been borne out by experience. We, therefore, add solid ammonium sulfate *after* ammonium hydroxide and sodium nitroprusside and just before comparison of test with standard.

How ammonium sulfate acts is unknown. Since it enhances color intensity with pure compounds in solution, it must participate directly in the chromogenic reaction. In this connection, when sulfate is added to 0.5 cc of an ammonium hydroxide, nitroprusside, SH mixture on a spot plate, it is possible to actually see color concentrating on each little crystal. This is not adsorption, but localized chemical reaction, since simple stirring disseminates the color throughout the mixture.

In view of the fact that denaturation of proteins may act to liberate SH groups,<sup>7</sup> and in view of the fact that ammonium sulfate is a protein precipitant, it might be that some of the enhancing effect in tissue and tissue extracts is derived from denaturation action as well as from direct influence of the compound.

on the test as such. If this is so, opportunity for variable results arises from the possibility that variations in kind and initial state of protein might be factors conditioning the amount of denaturation and hence the number of -SH groups liberated thereby. The matter deserves exploration.

*Zinc Acetate:* Swift fading of color produced in the nitroprusside reaction with -SH is a bar to exact work. Giroud and Bulliard<sup>4</sup> have proposed the use of 10 per cent zinc acetate as a color fixative, and results therewith have been reported.<sup>5-6</sup> The compound does act to prolong the duration of color and to fix it in tissues. But—and this is important—it also acts to decrease color intensity. Thus, when 0.1 c.c. of a 1 per cent solution of zinc acetate is added to 1.0 c.c. of a solution containing M/10,000 of any of the -SH carrying compounds used here—and the nitroprusside test is applied as hereinafter described—the color developed is never of the same order of magnitude as that of a control of the same initial -SH concentration (without acetate). Instead it is always much less, from 50 to 75 per cent mostly, sometimes even more. The reaction is not helped by varying the concentrations of the reagents.

This same inhibition of color intensity production occurs in tissue work. The color which is produced in tissue treated with zinc acetate and then well rinsed is never as good as that produced in similar tissue not treated; and what is more, color is found much more widely distributed in tissue not treated with acetate than in tissue exposed thereto. Thus, although a positive reaction in tissues treated with zinc acetate may indicate the presence of -SH therein, the absence of a positive reaction is no proof -SH is not there. In other words, preliminary treatment of tissues with zinc acetate may prevent demonstration of the true distribution of -SH therein by nitroprusside. It follows that despite the fact that duration of color is prolonged and color is fixed in the nitroprusside test for -SH by zinc acetate, its inhibitive and uncertain action on color intensity makes its use unsafe, either in qualitative demonstration or attempt at quantitative estimation.

*Cyanide: Sodium Potassium:* This reagent is supposed to show the presence of -S.S-. It is used to reduce disulfide to sulphydryl which is then demonstrated by nitroprusside. The assumption is made that the total -SH after reduction is the sum of the -SH present before reduction and that derived from -S.S- by cyanide action.

A negative reaction before and a positive reaction after cyanide is evidence that -S.S- is present and -SH absent. An increase in color intensity after cyanide, as contrasted with that given before, is suggestive but not conclusive as to the presence of -S.S-, unless the difference is large. For when the -SH concentration is low it is essentially impossible to discover whether the intensification derives from reduced -S.S- or from the high alkalinity of the dissociated cyanide. It sometimes happens that the color after reduction is less than that given before. It follows that the total -SH after reduction may not be the sum of the initial -SH and that derived from reduction of -S.S- by cyanide reduction. Indeed, in such cases, it is the sum of what initial -SH is left after standing in the highly alkaline cyanide medium and that formed by -S.S- reduction which has escaped destruction by the alkali. It might be here stated that -SH is destroyed on standing in highly alkaline solutions. We have

found more than once that whereas a root tip extract would show M/20,000 free SH, the color produced after six minutes reduction would show only M/100,000. In such cases it is impossible to tell if SS is present. This destroys all confidence in any reputed value of SS determinations by nitroprusside after cyanide reduction. All the method is good for is to sometimes establish the presence of -SS, but this with surety only when either no free SH is present or when the ratio disulfide/free SH is high.

It should now be evident that quantitative exactitude is impossible of attainment in the colorimetric estimation of SH via nitroprusside. On the other hand, a not too satisfactory approximation of the order of magnitude of free SH in solution can be had therewith if suitable precautions and patience are exercised.

Thus there is first prepared a M 1000 cysteine stock solution. From this a suitable range of standards (e.g., M/10,000 to M 100,000) is prepared by dilution. A short series is set up of 1 cc amounts in small test tubes. Then 1 cc of the solution to be tested, diluted tissue extract or juice is put into a similar test tube. To each tube is added 0.1 cc 27 to 29 per cent ammonium hydroxide and 0.1 cc 1 per cent sodium nitroprusside. After brief shaking to ensure mixing there is rapidly added to each tube 0.5 gm crystalline ammonium sulfate with quick shaking, and the test mixture is hastily matched with two standards to find which it is greater and which it is less than. The procedure is repeated with narrowing standard limits until the test range is established. With practice and experience it has been possible to tell whether test is closer to M/10,000 than to M/11,000, to M/50,000 than to M/52,000, to M 100,000 than to M/110,000, to M/200,000 than to M/250,000. That is to say with quick work one can place the free SH concentration in such material within 5 per cent of its detectable value in all except the very low limits of visible reaction.

Although this reads well and although the trained observer can get even better than 5 per cent placing with "pure solutions" of SH carrying compounds, it must not be forgotten that tissue extracts and other similar materials may contain many substances the action of which on the reagents and on SH itself is unknown. The estimation is at its best but an approximation. But as an approximation it has its uses.

Absolute values for free SH in fresh tissue slices are unobtainable. All that can be secured is an approximation of the relative concentrations in different parts of one and the same slice or region being looked at while the test is being applied. A useful procedure for gross work is to cover the tissue with 0.25 cc water, add 0.05 cc 27 to 29 per cent ammonium hydroxide and 0.05 cc 1 per cent sodium nitroprusside and then underline the tissue with 0.25 gm crystalline ammonium sulfate. For study under high power the amounts and the technique must be varied to suit the need. The order of introduction of the reagents into the test should however be just as described.

#### SUMMARY AND CONCLUSIONS

In vitro experiments made with the reagents of the nitroprusside test and highly purified SH yielding compounds show that each reagent offers variability factors. With these stabilized the test has a limited use in demonstrating the

presence of -SH and -S.S- under certain definite restrictions which are described in the text. It gives a useful approximation for free -SH. It has no quantitative value for -S.S- determination in cyanide-reduced material. It has no quantitative value, absolute or relative, in the presence of zinc acetate.

#### REFERENCES

1. Hopkins, F. G.: An Autooxidizable Constituent of the Cell, *Biochem. J.* 15: 296, 1921.
2. Kennaway, E. L., and Hieger, I.: Quantitative Studies of the Nitroprusside Reaction in Normal Tissues and Tumors, *Biochem. J.* 21: 751, 1927.
3. Pourbaix, Y., and Kennaway, E. L.: The Liberation From Yeast of Substances Giving the Nitroprusside Reaction, *Biochem. J.* 22: 1112, 1928.
4. Giroud, A., and Bulliard, H.: Sur une réaction des substances à fonction sulfhydryl, *Bull. Soc. chim. biol.* 14: 278, 1932.
5. Beck, K., and Urack, H.: Ueber die Arnold'sche reaction mit nitroprussidnatrium auf eiweissstoffe sowie über die denaturierung des fleischeiweisses durch konzentrierte harnstofflösung, *Ztschr. f. Untersuch. d. Lebensmitt.* 65: 399, 1933.
6. Chalkley, H. W.: The Chemistry of Cell Division. VII. The Distribution of Sulphydryl in Amoeba Proteus in Interkinesis and Mitosis, as Shown by the Nitroprusside Test, *Protoplasma* 28: 489, 1937.
7. Mirsky, A. E.: Coagulation of Myosin by Dehydration, *J. Gen. Physiol.* 20: 455, 1937.

## RELIABILITY OF THE AGGLUTINATION TEST FOR UNDULANT FEVER\*

WITH SPECIAL REFERENCE TO BRUCELLA AGGLUTININS IN TUBERCULOUS INDIVIDUALS

HOWARD J. SHAUGHNESSY, PH.D., CHICAGO, ILL., AND  
THOMAS C. GRUBB, PH.D., BALTIMORE, MD.

**D**IAGNOSTIC agglutination tests for undulant fever may be interpreted in several ways, since a positive test may indicate an active, latent, or past brucella infection, or a nonspecific reaction resulting from the presence of other infectious diseases.<sup>1, 2</sup> Amoia<sup>3</sup> has recently demonstrated experimentally that the anamnestic phenomenon may account for the finding of brucella agglutinins in the presence of other infectious diseases.

According to Topley and Wilson<sup>4</sup> the sera of normal persons may contain brucella agglutinins in a titer of from 1:50 to 1:100, though the status of a "normal" person regarding exposure to brucella infections is rather precarious in view of the now well-recognized widespread incidence of Bang's disease. The reports of Carpenter, Boak, and Chapman,<sup>5</sup> Evans<sup>6</sup> and Scoville<sup>7</sup> make it evident that a negative brucella agglutination test by no means excludes the possibility of even an active brucella infection. To add to the confusion, various workers are not agreed upon the agglutination titer which should be considered of diagnostic significance.<sup>2</sup> Lack of uniform technique in performing the agglutination test is probably responsible for this disagreement upon what constitutes a significant diagnostic titer.<sup>8</sup>

\*From the Division of Laboratories, Illinois State Department of Public Health, Chicago, Illinois, and the University of Maryland School of Pharmacy.

Received for publication, March 14, 1938.

It is thus apparent that the clinical significance of the results of a brucella agglutination test is equivocal. This is particularly unfortunate, since the protean symptoms of undulant fever may be easily confused with those of many other infectious febrile diseases<sup>9</sup> and in many cases the physician is forced to depend entirely upon a laboratory test, usually the agglutination test, for the final diagnosis.

Our attention was first drawn to the problem of the reliability of the agglutination test for undulant fever some time ago when it was found that a small percentage of blood samples sent to the state health department laboratories from a municipal tuberculosis sanatorium\* contained brucella agglutinins† in a high titer. Since, so far as we are aware, no study had been made in this country of the possibility of falsely positive brucella agglutination tests being obtained with the sera of tuberculous individuals, it was decided to investigate this problem. The method employed in this work gave us information regarding the general reliability of both the positive and negative brucella agglutination tests, as well as data on the presence of brucella agglutinins in tuberculous sera.

#### METHOD OF STUDY

From 1930 to 1935 positive brucella agglutination tests were obtained with the sera of 405 patients submitted to the main laboratories of the Division of Laboratories of the Illinois Department of Public Health. It was believed that if the number of patients who had any evidence of tuberculosis and no symptoms of undulant fever could be determined the possible serologic relationship of these two diseases or the presence of nonspecific agglutinins might be detected. Accordingly, a carefully worded questionnaire was sent to all physicians who had submitted blood samples giving a positive brucella agglutination test during this five year period. The information requested in this questionnaire was as follows:

Patient's name, age, sex and race

Occupation at the time the positive agglutination test was obtained

Final clinical diagnosis—was it undulant fever? (Answer yes or no)

Did the patient have typhoid fever, paratyphoid fever, tuberculosis, or tularemia six months before or after the positive brucella agglutination test was found?

It will be noted that we have purposely included several diseases other than tuberculosis in the last question, so that the clinician would gain no clue as to the purpose of this questionnaire. Information was obtained concerning 327 of the 405 patients, the remaining number being unaccounted for through death of the physician, failure to make a clinical follow up, or failure to return the questionnaire.

*Reliability of the Positive Undulant Fever Agglutination Test*—The information obtained from the returned questionnaires is summarized in Table I.

\*We are indebted to Dr. M. Pollak, Medical Director of the Peoria Municipal Tuberculosis Sanatorium, for sending us blood samples and data on the physical findings and diagnosis of the patients.

†For the purpose of this report we have assumed that any serum which produces a positive brucella agglutination test contains brucella agglutinins though we realize that this is not necessarily true for agglutination of the brucella antigen may take place in the absence of specific brucella agglutinins.



TABLE I

CORRELATION OF CLINICAL DIAGNOSIS WITH RESULTS OF THE POSITIVE AGGLUTINATION TEST\*

		Per cent
No. of patients with a positive agglutination test	327	
Positive agglutination test confirmed by clinical findings	266	\$1.34
No. of patients in whom the diagnosis of undulant fever was uncertain	13	3.97
Positive agglutination test not confirmed by clinical findings	16	4.89
Positive agglutination in patients with typhoid fever	4	1.22
Positive agglutination in patients with tuberculosis	25	7.64
Positive agglutination in uncertain cases of tuberculosis	3	0.90
Positive agglutination in patients with paratyphoid fever	0	
Positive agglutination in patients with tularemia	0	

\*In this paper only agglutination tests with a titer of 1:160 or above are included. However, it is our policy to report to the physician a titer of 1:80 as a "doubtful positive" and request another specimen. Tests with titers below 1:80 are reported as "negative."

The table indicates that in slightly over 80 per cent of the cases the clinical diagnosis agreed with the results of the agglutination test. If the physician was uncertain about the diagnosis, he was asked to indicate this fact in the questionnaire. As the table shows, the diagnosis was uncertain in about 4 per cent of the brucella cases and in 0.90 per cent of the tuberculosis cases. The number of these uncertain diagnoses is not large enough to affect any general conclusions which may be drawn from the data.

It should be especially noted that the sera of 25 tuberculous individuals, all from the above-mentioned tuberculosis sanatorium, gave positive brucella agglutination tests, the titers ranging from 1:160 to 1:2560. The possibility that these positive tests were false or nonspecific results will be discussed in one of the following sections. If we assume for the moment that these tuberculous patients also had unrecognized, latent, or past cases of undulant fever, then the reliability of the positive agglutination test becomes 88.50 per cent; if we omit these 21 cases altogether, then the reliability of the positive test becomes 88.98 per cent.

Since the original report of Francis and Evans,<sup>10</sup> it is usually stated in the literature that there may be a cross agglutination between brucella and tularemia agglutinins. It is, therefore, of interest to note that of the 327 sera containing brucella agglutinins, not one was reported to have come from a patient with tularemia, although from 130 to 150 cases of tularemia are reported in this state annually. Only a negligible percentage of persons with typhoid fever and none with paratyphoid fever gave a positive brucella agglutination test. The number of persons having a positive brucella agglutination test, who were reported definitely not to have had brucellosis, was 16, or 4.89 per cent of the total number of cases. It appears, therefore, from our data, that a positive brucella agglutination test may be relied upon to indicate clinical undulant fever in about 81 per cent of the cases; if there is no suspicion of tuberculosis, a positive test is reliable in about 89 per cent of the cases.

*Reliability of the Negative Agglutination Test.*—Since it is obviously of equal clinical importance to know the reliability of the negative as well as the positive agglutination test for undulant fever, we prepared a suitable questionnaire to obtain information regarding the clinical diagnosis of patients whose sera gave a negative agglutination test. This questionnaire, requesting the following

information, was sent to all physicians who had submitted, during the past six months, sera giving a negative brucella agglutination test.

Patient's name, age, and sex.

Final clinical diagnosis.

When no diagnosis was made, any of the following signs or symptoms that the patient had were to be checked.

Fever (continuous or intermittent), weakness, pains (location), sweating, chills, loss of weight or appetite, nervousness, constipation.

Additional information supplementing the above data was also solicited.

The purpose of this questionnaire was to determine the correlation between the negative agglutination test and the clinical diagnosis. In those cases where the diagnosis was uncertain, it was hoped that by checking any of the most frequently observed signs or symptoms listed by Hardy and co-workers<sup>11</sup> we might be able to detect an unsuspected case of undulant fever. Information regarding the clinical diagnosis of 358 patients was obtained and is summarized in Table II.

TABLE II

CORRELATION OF CLINICAL DIAGNOSIS WITH RESULTS OF THE NEGATIVE AGGLUTINATION TEST

No. of patients with a negative agglutination test	358
Negative agglutination test confirmed by clinical findings	281 (86.2 per cent)
Negative agglutination test in patients with a clinical diagnosis of undulant fever	
a. Sera obtained less than two weeks after onset	3
b. Sera obtained later than two weeks after onset	5
c. Date of onset not given	11
	19 (5.8 per cent)
No. of patients without a definite clinical diagnosis but with symptoms suggestive of undulant fever	26 (8.0 per cent)

Our results indicate that in at least 86 per cent of the cases a negative agglutination test substantiated the physician's diagnosis. In 5.8 per cent of the cases the physician stated that the patient had undulant fever. It will be noted, however, that in at least three of these cases the blood sample was submitted before agglutinins might have had time to develop. It is also probable that this time factor was not considered in some of the cases where no date of onset was recorded by the physician. Some 8.0 per cent of the patients, for whom the physician could give no definite diagnosis, had symptoms suggestive of undulant fever, but we naturally hesitate to hazard a guess as to how many of these patients were actually suffering from clinical undulant fever. From a general consideration of the requested and supplementary information obtained from these questionnaires, and from our own previous experience, we feel that in our hands the negative agglutination test for undulant fever will agree with the clinical diagnosis in at least 90 per cent of the cases.

*Cross Agglutinations*—The serologist performing diagnostic tests is ever mindful of the possibility of cross agglutinations as a source of false positive tests. We considered it of more than academic interest to analyze our data for the purpose of determining just how seriously cross agglutinations affect the reliability of the brucella agglutination test. Our laboratory records show that

out of the 314 sera submitted for typhoid and brucella agglutination tests, 5, or 1.6 per cent, of the sera gave cross reactions. Out of 394 sera submitted for brucella and *B. proteus* X19 agglutination tests, 13, or 3.3 per cent, showed cross agglutinations. Of the 71 sera examined for paratyphoid A and brucella agglutinins, 74 for paratyphoid B and brucella agglutinins, or 14 for tularemia and brucella agglutinins, no cross agglutinations were observed. Thus, out of the 867 sera examined for brucella and other agglutinins only 18, or approximately 2 per cent, showed cross agglutinations. It should be emphasized that even these so-called cross agglutinations might have been true agglutinations due to the presence of past, latent, or active brucella infections in patients with typhoid, paratyphoid fever, etc. In practice where such cross agglutinations occur it is frequently necessary to examine several samples from the patient before determining which agglutinins are of diagnostic significance.

Before discussing the significance of a positive brucella agglutination test in tuberculous individuals, we believe it is important to outline briefly the technique of the brucella agglutination test performed in our laboratories during the period covered by this study.

#### TECHNIQUE OF AGGLUTINATION TEST

A polyvalent antigen of 5 brucella strains (*B. abortus* No. 456 and *B. melitensis* No. 428 from the National Institute of Health; *B. melitensis* No. 304 and *B. abortus* No. 229 from the University of Illinois; *B. suis* No. 71 from the University of Chicago) is prepared by growing these strains separately on Difco liver infusion agar for forty-eight hours at 37° C. The growth of each strain is washed off the slants with 0.85 per cent sodium chloride, and the turbidity of each suspension is standardized to conform with a No. 3 McFarland barium sulfate nephelometer. Equal parts of each suspension are then mixed and enough formalin added to give a final concentration of 0.05 per cent. In performing the test, 0.25 c.c. of the antigen is added to 0.25 c.c. of the diluted serum (final serum dilutions of 1:40 to 1:1280 are routinely used) in each agglutination tube, and the tubes then incubated four hours in a 37° C. water bath. The tubes are next removed from the water bath and placed in the 37° C. incubator for eighteen to twenty-four hours before reading the tests. All of the agglutination tests reported in this paper were performed by one individual.

Since the finding of falsely positive brucella agglutination tests has frequently been attributed to the use of strains containing a rough antigen, we tested the thermoagglutination of all of our strains according to Burnet's<sup>12</sup> technique. None of the strains used in our brucella agglutination tests showed any evidence of thermoagglutination, which is usually interpreted as indicating the absence of rough antigen. Although it seems to be now generally agreed that "no advantage is obtained through the use of separate or combined antigens" (Huddleson<sup>13</sup>) and that an antigen made of *B. abortus* is satisfactory for detecting agglutinins produced by all three species of brucella, we have continued to use the polyvalent antigen since it has given us such satisfactory results. In this connection it may be of interest to note that in a recent publication by Higginbotham and Heathman<sup>14</sup> they state that "The results support the contention that a polyvalent antigen including some local strains should be employed in the routine serological examination for undulant fever."

## BRUCELLA AGGLUTININS IN TUBERCULOUS INDIVIDUALS

So far as we know, there has been no specific study made in this country to determine the possibility of finding falsely positive or nonspecific brucella agglutinins in the sera of tuberculous persons. In the recent report of Stone and Bogen<sup>15</sup> concerning the incidence of undulant fever in three tuberculosis sanatoriums, it was tacitly assumed that a positive agglutination test in the tuberculous inmates indicated an active, latent, or past brucella infection. If the possibility of nonspecific agglutinations had been considered in their work, the discrepancies between the skin tests and the agglutination tests, and the apparently rapid disappearance of brucella agglutinins might not have been so puzzling.

Within recent years several Italian workers have studied the incidence of brucella agglutinins in the sera of tuberculous persons. Magliulo<sup>16</sup> found agglutinins for *B. melitensis* and *B. paramelitensis* in the sera of 30 of the 60 patients examined. He concluded that these results were explained by the fact that the tubercle bacillus and *B. melitensis* and *B. paramelitensis* contained a common group antigen. The validity of Magliulo's conclusions is questionable, since he did not test his brucella strains for the presence of a rough antigen, and sera from many patients with nontuberculous diseases, such as endocarditis, psoriasis, puerperal fever, syphilis, carcinoma, etc., also agglutinated *B. melitensis* in titers of 1:100 to 1:150. Sanfilippo<sup>17</sup> examined the sera of 132 tuberculous patients to detect agglutinins for *B. melitensis* and *B. paramelitensis*. The *melitensis* strains were agglutinated only by the sera of two patients not showing clinical brucellosis, but many of the sera contained agglutinins for *B. paramelitensis*. One of the *paramelitensis* strains was agglutinated by 33 of the sera, another by 76, another by 80 and the fourth strain was agglutinated by 103 of the 132 sera. On the other hand, Morellini<sup>18</sup> did not find any agglutinins for *B. melitensis* or *B. abortus* in the sera of 60 open cases of tuberculosis. He believed that the positive results obtained by others were due to the use of old strains for antigens or that there were dual infections of tuberculosis and undulant fever.

The relationship between brucella and tuberculous infections was studied by Sarnowicz<sup>19</sup> through the use of the allergic reaction in guinea pigs, cattle, and human beings. He found that tuberculous animals frequently gave a positive reaction to abortin, but animals infected with *B. abortus* reacted slightly, or not at all, to tuberculin.

The meager and conflicting literature on the problem of brucella agglutinins in tuberculous persons does not permit any general conclusions to be drawn at the present time. It is hoped, however, that this report will offer additional information and stimulate further investigation of the problem in this country.

Between 1930 and 1935 our laboratories performed brucella agglutination tests on blood samples from approximately 650 patients shortly after they were admitted to the municipal tuberculosis sanatorium previously referred to. No brucella agglutinins were found in 629, or 96 per cent, of these sera. The majority of the 25 patients whose sera gave a positive agglutination test on the first examination, gave an entirely negative result when their sera was retested after a short interval. For example, one patient's serum showed a titer of

1:2560 on the first test, and four days later no brucella agglutinins could be demonstrated in the serum of this same patient. Another patient had a titer of 1:1280 on the first test, and seven days later the test was completely negative. These puzzling results were obtained despite meticulous care to maintain a uniform technique through the use of the same antigen, dilutions, incubation, etc. It was noted that in the majority of these agglutinations with tuberculous sera, the agglutination was of the finely granular type.

In order to determine whether or not we would obtain similar results from another tuberculosis sanatorium, samples of blood were obtained from 102 patients at the Chicago Municipal Tuberculosis Sanatorium.\* Approximately 9 per cent of these sera gave positive brucella agglutination tests; and the majority of these sera showed a much lower titer or were completely negative when retested seven to ten days later. Eight of the patients whose sera gave a positive brucella agglutination test and 14 patients whose sera gave a negative test were given an intradermal injection of "Brucin."† None of these patients showed a positive skin test. We also obtained sera from 28 patients in another county tuberculosis sanatorium, but none of these gave a positive agglutination test.‡

The milk used at the Peoria institution was certified raw milk. The agglutination tests, however, were performed within a few days after the patients had been admitted, so that even if there were some undetected cows infected with Bang's disease in the herd, it is very improbable that the patients had become infected and owed their agglutinins to this source. Carpenter, Boak, and Chapman<sup>5</sup> reported that brucella agglutinins are not passively transferred in milk, so that the presence of brucella agglutinins in the patients shortly after their admission probably could not be accounted for on the basis of passive transfer through the drinking of milk containing brucella agglutinins. Pasteurized milk is supplied to the patients at the Chicago sanatorium, so that there is also probably no chance for infection through this route in this institution.

The results of our investigation indicate that the sera of a small percentage of tuberculous individuals may show a transitory brucella agglutinin content. We believe that these agglutinations should be considered as false positive or nonspecific results since it is inconceivable that an agglutination titer of 1:2560 or 1:1280 should completely disappear within the course of a few days, if we are dealing with a true brucella infection. The well-known phenomenon of the disappearance of antibodies from the blood stream in the agonal period is not pertinent here. The absence of a positive skin test in those patients tested is considered further evidence that we were dealing with a nonspecific agglutination phenomenon. It is not believed that the hypotheated common group antigen of the tubercle bacillus and the brucella organisms could explain our re-

\*We wish to express our indebtedness to Dr. Allan J. Hruby and Dr. H. C. Sweany for supplying us with samples of blood.

†The Brucin was obtained through the courtesy of Dr. I. F. Huddleson, and the skin tests were performed by Dr. M. R. Lichtenstein of the Chicago Municipal Tuberculosis Sanatorium.

‡We are indebted to Dr. D. O. N. Linberg, Medical Director of the Macon County Tuberculosis Sanatorium for sending us these sera.

sults, for if such were the case, we would not expect the complete disappearance of agglutinins within a short time. It is possible that in those cases where the agglutination titer did not disappear upon repeated testing, the tuberculous patient also had an unrecognized case of latent or past undulant fever contracted before entering the institution. Since a recent survey by the Bureau of Animal Industry of the United States Department of Agriculture\* showed that from 20 to 30 per cent of the cattle in Illinois have Bang's disease, and Torrey and Graham<sup>20</sup> isolated brucella organisms from 50 per cent of 62 raw milk samples collected in widely distributed counties in Illinois, it is quite possible that these patients obtained their brucella infection before entering the institutions.

#### BRUCELLA AGGLUTININS IN TUBERCULOUS GUINEA PIGS

In order to determine if the presence of brucella agglutinins could be demonstrated in animals experimentally infected with tubercle bacilli, the following study was carried out.

The two weeks' growth of a stock strain of the human tubercle bacillus on Petriagar medium was washed off with saline and ground in a mortar to obtain a homogeneous suspension. Approximately 500 000 tubercle bacilli were injected subcutaneously into the left inguinal region of 25 guinea pigs. Samples of blood were taken from each animal by cardiac puncture before and at intervals of two weeks after inoculation of the tubercle bacilli. It was originally planned to keep the animals that did not die of tuberculosis for a period of three months, but due to the excessive summer heat (105° to 110° F. in the animal quarters), all of the animals died about nine weeks after inoculation. Using the technique previously outlined, the sera from these animals were examined for the presence of brucella agglutinins. All animals were autopsied for gross evidences of tuberculosis, and smears made of the affected organs to detect the presence of tubercle bacilli.

None of the animals showed brucella agglutinins in their sera before injecting the tubercle bacillus, but four showed agglutinins after the inoculation. Table III shows the time and titer of the agglutinins found in these animals.

TABLE III

POSITIVE BRUCELLA AGGLUTINATION TITERS IN SERA OF GUINEA PIGS INOCULATED WITH HUMAN TUBERCLE BACILLI

ANIMAL NO	TIME INTERVAL AFTER INOCULATION WHEN BLOOD WAS TAKEN			
	2 WEEKS	4 WEEKS	6 WEEKS	8 WEEKS
643	0	1 80	0	0
A 18	1 40	1 160	0	1 640
637	1 40	1 160	0	0
579	1 40	0	0	0

These results lend experimental confirmation to our findings in human beings, for brucella agglutinins were found in the sera of tuberculous guinea pigs, and the agglutination titers were irregular and of a transient nature. It is interesting to note that in seven of the guinea pigs, no gross evidences of tuberculosis were observed, and no tubercle bacilli were found in stained smears of lymph nodes, spleen, liver, and lungs. It is possible that the infection had not advanced far enough in nine weeks to be detected by our methods of examination.

\*Report not yet published.

Of the four animals showing brucella agglutinins, only one (A-18) showed the pathologic changes of tuberculosis. Apparently a progressive infection is not necessary to cause the production of nonspecific agglutinins for brucella organisms.

#### DISCUSSION

Since the agglutination test for undulant fever has not yet had the test of time in this country that the Widal test and other serologic procedures have, the reliability of its results is frequently questioned. However, our study has revealed the fact that a positive test may be relied upon in 81, and perhaps 89, per cent of the cases, and the negative test in about 90 per cent of the cases. This is a high degree of reliability when it is realized that in a recent study on the serologic diagnosis of syphilis conducted by the United States Public Health Service and carried out by many of the country's leading serologists, it was found that positive results were obtained in only 65 to 88 per cent with known syphilitic sera.<sup>21</sup> It may be objected that our method of determining the reliability of the brucella agglutination test is inherently fallacious, since, as we have already pointed out, the physician is frequently forced to base his final diagnosis solely on the laboratory examination of the patient's blood. We do not believe, however, that this criticism is valid, since in the majority of cases the clinical information was obtained from the physician several months, and in most instances several years, after the positive agglutination test was reported. Thus there was usually ample time to detect and diagnose other diseases which might have been mistaken for undulant fever.

Our results indicate that cross agglutinations with serum containing agglutinins for *B. typhosum* or *B. proteus* X19 may be expected in about 4 per cent of the cases, but this does not constitute a serious limitation to the reliability of the test, as subsequent blood samples from the patient in question will usually show an increase in titer of agglutinins for the infectious agent causing the illness.

From our present work it appears that the sera from tuberculous individuals may produce a positive brucella agglutination test at desultory intervals. This finding is supported by the results obtained with experimentally infected guinea pigs.

The phenomenon of nonspecific agglutination by tuberculous sera is not new, for Hull and Henkes<sup>22, 23</sup> reported it to occur with *B. typhosum* and *B. dysenteriae* (Flexner). Hull and associates<sup>24</sup> also found that several members of the paratyphoid group were agglutinated by sera from tuberculous persons. Apparently there is some nonspecific agent in tuberculous serum which, under certain conditions, is capable of causing the agglutination of a variety of bacterial antigens.

#### SUMMARY

1. In order to determine the reliability of the agglutination test for undulant fever, information concerning the symptoms and clinical diagnosis of patients whose sera had been examined for brucella agglutinins was obtained through questionnaires sent to the attending physicians.

2 The results of the positive agglutination test for brucellosis agreed with the clinicians' diagnosis in 81 to 89 per cent of the cases, the negative agglutination test was substantiated by the clinical diagnosis in about 90 per cent of the cases

3 An analysis of our data indicated that cross agglutinations were obtained in about 2 per cent of the sera containing agglutinins for *B. typhosum* or *B. proteus* X19. The possibility that these results may not have been due to true cross agglutinations is discussed

4 *Brucella* agglutinins were found in approximately 3 per cent of 650 sera from the patients in one municipal tuberculosis sanatorium and in 9 per cent of the 102 sera from the patients in another tuberculosis sanatorium. The transient and irregular behavior of these agglutinations and the absence of positive skin tests indicated that they were probably nonspecific or false reactions

#### REFERENCES

- 1 Hauptman W. Beweis ein positives Agglutinationsergebnis das Vorhandensein von Bunscher Krankheit Med Klin 31 1174 1925
- 2 Gray, J. D. A. The Significance of Brucellar Agglutinins in Human Sera J Bact 25 415 1933
- 3 Amour, R. Observations sur l'agglutination par les 'Brucelles' Soc internaz di microbiol, Boll d sez ital 5 171, 1932
- 4 Topley, W. W. C., and Wilson, G. S. Principles of Bacteriology and Immunity, Baltimore, 1929, William Wood & Co
- 5 Carpenter, C. M., Borch, R., and Chapman O. D. The Significance of Brucella Abortus Agglutinins in Human Serum, J Immunol 17 65 1929
- 6 Evans A. Chronic Brucellosis J A M A 103 667 1914
- 7 Seaville, W. B. The Prevalence of Mild Brucella Abortus Infections J A M A 105 1976, 1935
- 8 Evans A. Laboratory Procedures for the Diagnosis of Undulant (Malta) Fever, Am J Pub Health 17 399, 1927
- 9 Hasseltine H. E. Current Studies of Undulant Fever Pub Health Rep 41 1659, 1929
- 10 Francis, E., and Evans, A. Agglutination, Cross Agglutination, and Agglutinin Absorption in Tularemia, Pub Health Rep 41 1273 1926
- 11 Hardy, A. V., Jordan, C. F., Borts, I. H., and Hardy, G. C. Undulant Fever, Nat Inst Health Bull No 158, 1931
- 12 Burnet, R. La Thermo agglutination et l'evolution de l'espece Brucella, Arch Int Pasteur de Tunis 17 128, 1929
- 13 Huddleson, I. F. Brucella Infections in Animals and Man New York 1934 The Commonwealth Fund
- 14 Higginbotham, M., and Heathman L. S. Precipitin and Complement Fixation Reactions of Polysaccharide Extracts of Brucella, J Infect Dis 59 30 1936
- 15 Stone, R. V., and Bogen, E. Studies of Correlated Human and Bovine Brucellosis, Am J Pub Health 25 580, 1935
- 16 Magliulo, L. Siero reazione di Wright e Tuberculosis, Gior di batteriol e immunol 10 284, 1933
- 17 Sanfilippo, E. Potere Agglutinante del Siero dei Tubercolotici sui Germi del Gruppo Brucella, Gior di batteriol e immunol 11 1, 1933
- 18 Morellini, M. La Reazione di Wright per il Micrococco di Bruce sul Siero di Sangue dei Tubercolotici, Riforma med 49 1697, 1933
- 19 Sarnowicz, W. Contribution a l'Etude des Reactions Allergiques Cousees dans la Tuberculose et la Brucellose, Ann Inst Pasteur 53 106 1934
- 20 Torrey, J. P., and Graham, R. Brucella Abortus in Market Milk, Vet Med 31 61, 1936
- 21 Cumming, H. S., Hazen, H. H., Sanford, A. H., Senevir, F. E., Simpson W. M., and Vonderlehr, R. A. The Evaluation of Serodiagnostic Tests for Syphilis in the United States Ven Dis Inform, Suppl No 1, 1935
- 22 Hull, T. G., and Henkes, K. The Widal Test in Tuberculosis, Am Rev Tuberc 8 266, 1923
- 23 Hull, T. G., and Henkes, K. The Agglutination of the Flexner Dysentery Bacillus by the Blood Serum of Tuberculous Persons, Am Rev Tuberc 8 272 1923
- 24 Hull, T. G., Henkes, K., and Cassidy, H. Agglutination Reactions of the Paratyphoid Dysentery Group in Tuberculosis, Am Rev Tuberc 11 79, 1925



## THE IRON OF HUMAN BLOOD SERUM\*

BURNHAM S. WALKER, M.D., BOSTON, MASS.

IN TEXTBOOKS of physiologic or clinical chemistry it is usually stated, or tacitly assumed, that the blood contains iron in the form of hemoglobin, and in that form only. On this assumption is based the frequent practice of hemoglobin estimation in blood on the basis of the iron content. That this procedure gives a very close approximation to the correct hemoglobin value is attested by the reports of large series of comparative determinations by several competent investigators.<sup>1-4</sup>

Iron has been recognized in the blood in forms other than hemoglobin. The credit for this discovery should probably go to Biernacki,<sup>5</sup> although the work of Erben<sup>6</sup> and of Fowell<sup>7</sup> is more frequently cited. All these investigators were hampered by the lack of adequate analytical methods, and by the current uncertainty as to the iron content of hemoglobin. This latter difficulty was resolved in 1909 by Butterfield;<sup>8</sup> Fowell apparently did not have the benefit of his figures.

Of present-day investigators, Barkan<sup>9</sup> has been outstanding in his contributions to our knowledge of nonhemoglobin iron. He has demonstrated that a portion of the iron in the red disks differs from hemoglobin iron in being "leicht abspaltbar"—easily separated, or labile to dilute mineral acids. Iron in a similarly labile combination is present in plasma or serum. The labile iron is present in quantities independent of the hemoglobin content of the blood sample. About 65 per cent of the labile iron (fraction E) reacts reversibly with oxygen and carbon monoxide; the remainder (fraction E') does not. In Barkan's most recent publication, he offers evidence that the labile iron consists of:

- I.  $\alpha$ -Pseudohemoglobin, derived from hemoglobin by rupture of the porphyrin ring (fraction E)
- II.  $\alpha$ -Pseudomethemoglobin, derived from I by oxidation of the ferrous to ferric iron (fraction E')
- III. Iron liberated from II along with globin and bilirubin by reduction. This iron is liberated into the plasma where it forms a compound with plasma globulin. This fraction forms a major portion of the plasma or serum iron.

Barkan's hypothesis is strongly confirmed by the observed fact that iron and bilirubin are present in normal human plasma in equimolecular proportions. Increase in blood serum iron with bilirubinemia has been independently reported by Locke, Main, and Rosbash.<sup>10</sup>

\*From the Evans Memorial for Clinical Research and Preventive Medicine, Boston.  
Received for publication, March 23, 1938.

The nonhemoglobin fractions of blood non dealt with by Warburg and Kiebs<sup>11</sup> and by Klumpp<sup>12</sup> may be considered as comparable, if not identical, with the labile non of Barkan. Klumpp found 2.2 mg of nonhemoglobin iron as a mean value in whole blood, making up a mean percentage of 5.3 of the total non. His thesis is that the variability of this nonhemoglobin non (in both cells and plasma) precludes the possibility of accurate hemoglobin analysis by any non method. Josephs<sup>13</sup> found somewhat more nonhemoglobin non (3 mg per 100 cc) in whole blood, and more recently Josephs and Winocur<sup>14</sup> have demonstrated that this nonhemoglobin fraction of non in the red disks is increased under certain conditions without a simultaneous increase in the labile iron of Barkan. This brings up the possibility of still other fractions of non hemoglobin non, which await more definite characterization. Moore<sup>15</sup> has recently summarized the literature on the quantitative determination of the labile non in whole blood and given the results of his own analyses which show that from 2.4 to nearly 10 per cent of the non in normal whole blood is "easily split off," the percentage apparently depending somewhat upon the mineral acid used, and the method of separation.

This study deals with the iron of human blood serum, which consists, according to Barkan's latest formulation, chiefly of ferric non in combination with serum globulin. This concept agrees with the observed facts that serum non is not ultrafiltrable unless acidified,<sup>9</sup> is partially removed by precipitation of the proteins,<sup>16</sup> but still gives some of the reactions of inorganic iron.<sup>17</sup> Moore<sup>15</sup> has shown that the amounts of non in serum and plasma are essentially identical, provided the separation is made within an hour. Longer standing of blood before separation will result in a measurable transfer of non from cells to plasma or serum.<sup>9</sup> Moore, Doan, and Arrowsmith<sup>18</sup> assign to plasma non the function of transport; it represents an equilibrium of rates of iron intake, utilization, and excretion.

Several methods have been proposed for the measurement of serum or plasma iron. The possibility of contamination with hemoglobin has occurred to all investigators of this fraction, and several procedures have been used to eliminate it. Bing and Hanzal<sup>19</sup> corrected their values by carrying out a determination of hemoglobin by the quantitative benzidine method of Bing.<sup>20</sup> Moore<sup>15</sup> used a similar procedure. The use of benzidine in this manner is open to the objection that benzidine is not a specific reagent for hemoglobin, but responds to any substance which has a peroxidase action, including non itself in the ferric state. A solution of ferric pyrophosphate of the same concentration of iron as normal human blood serum (0.1 mg per 100 cc) will give a detectable blue color with the benzidine reagent used by Moore. In the majority of Moore's analyses the correction was of the order of 1 per cent of the total serum non, which is insignificant considering the error of the iron analysis itself. Other workers have used trichloroacetic acid filtrates of serum or plasma. With this method hemoglobin is precipitated and cannot interfere. The objection to the use of trichloroacetic acid is that a variable amount of nonhemoglobin non is removed in the precipitation, this was first noted by Fowweather<sup>21</sup> and confirmed by Moore.<sup>15</sup> In view of the unsatisfactory outcome of attempts to cor-

rect for the very small amounts of hemoglobin possibly present, the figures in this study are not corrected: any specimens showing visible or spectroscopic evidence of hemolysis have been excluded.

The first experimental phase of this study was concerned with the development of a method permitting the examination of sera with accuracy and reasonable speed. The method developed was a modification of that of Fowweather.<sup>16</sup> It differs from the original method in the following respects: serum is used rather than oxalated plasma; the use of platinum is obviated; complete oxidation to the ferrie state is assured by the use of persulfate; the color is more stable; the Evelyn photoelectric colorimeter is used for readings.

*Procedure for Serum Iron.*—Two cubic centimeter samples of unhemolyzed serum are digested in pyrex test tubes (graduated at 20 c.e.) with 1.5 c.e. of concentrated sulfuric acid. A glass bead is adequate to prevent bumping. The digestion may be carried out slowly on a sand bath or rapidly by the direct flame of a microburner. When the mixture is well charred, 30 per cent hydrogen peroxide is added in 0.5 c.e. amounts, repeating as often as necessary, with continued heating, to produce a solution which remains clear and colorless. Usually three additions are required. A control tube containing 1.5 c.e. of concentrated sulfuric acid should be carried along with the others and should receive the same amount of peroxide. When the contents of the tubes is clear and cooled to room temperature, the volume is made up to 20 c.e., and to each tube is added 1 c.e. of saturated potassium persulfate solution and 4 c.e. of 3 N potassium thiocyanate solution. These two solutions are prepared as prescribed by Wong.<sup>21</sup> The colored solutions may be read in an ordinary colorimeter against a standard prepared by using 0.5 c.e. of a standard iron solution containing 0.01 mg. iron per c.e., made up in a similar tube to 20 c.e. volume, and to which identical amounts of persulfate and thiocyanate solutions have been added as to the unknowns. The Evelyn photoelectric colorimeter obviates the difficulty of comparing by eye readings the rather faint colors that are developed. The optimum filter for iron thiocyanate colorimetry would be at 480 m $\mu$ .<sup>22</sup> The 520 m $\mu$  filter used for creatinin determinations has given entirely reproducible results.

*Normal Values for Serum Iron.*—Blood sera from 12 medical students were examined. The highest value found was 0.23 mg. iron per 100 c.e. serum, the lowest value 0.04 mg., the mean value 0.13 mg.

Comparison of these values with those of Moore<sup>15</sup> and of other investigators as tabulated in his recent paper shows that there is fairly general agreement in regard to the normal values for human blood serum iron. Our maximum value is somewhat higher than that observed by Moore, agreeing more closely with that of Tompsett;<sup>27</sup> our minimum is very slightly lower than Moore's, and in agreement with Guthmann, Brückner, Ehrenstein, and Wagner.<sup>23</sup> Recently, Jenkins and Thompson,<sup>29</sup> using the thioglycollic acid method, reported much more iron in the plasma than is generally accepted, and also a marked sex difference unconfirmed by other workers. The most extensive report on blood serum iron published to date is that of Heilmeyer and Plötner.<sup>30</sup> They find an average value in males of 0.126 mg. per 100 c.e., which is in agreement with our average value of 0.13 mg. They also observed a sex difference, but it was small and opposite in direction to that reported by Jenkins and Thompson.

*Serum Iron in Pernicious Anemia.*—The suggestion that the nonhemoglobin iron of the blood serum might be increased in pernicious anemia is an old one. It was first expressed by Erben,<sup>6</sup> later by Fowell,<sup>7</sup> Riecker<sup>25</sup> and Marlow and Taylor<sup>26</sup> investigated the question, but came to conflicting conclusions. Locke, Main, and Rosbush<sup>10</sup> studied 3 cases of pernicious anemia and found high values (not beyond normal limits) which decreased under liver therapy. Moore, Doan, and Arrowsmith<sup>18</sup> tabulated 10 cases of pernicious anemia, of which 5 showed serum iron values above normal. Heilmeyer and Plotner<sup>30</sup> classify pernicious anemia among the hemolytic anemias, reporting 8 cases. Only one untreated case shows a value above their normal range, but all are high in the normal range, and show a decrease in response to liver therapy. The same lowering of serum iron levels following treatment is demonstrated by Moore, Doan, and Arrowsmith; in one case where liver was temporarily discontinued there was a return to a high value.

This present study adds two untreated cases and several treated cases of pernicious anemia.

Mr. McL., aged 46 years, entered the hospital with 36 per cent hemoglobin and 1.6 million red blood cell count; his serum iron was 0.27 mg. the day before liver therapy was begun. Subsequent values are shown in Table I.

C., aged 36 years, was admitted with 27 per cent hemoglobin and 1.01 million red blood cell count; her serum iron on admission was 0.30 mg. Her course is shown also in Table I.

TABLE I

DAY OF TREATMENT	SERUM IRON (MG. PER 100 C.C.)	
	McL.	C.
2	0.28	
5	0.26	
9	0.18	
12	0.22	
13		0.11
15		0.13
16	0.14	
18		0.12
19	0.09	
22	0.10	0.09
29	0.08	
31		0.08
42		0.08
59		0.13
87		0.14

Fifteen serum iron determinations have been made in cases of pernicious anemia under treatment; of these, only two have been above the normal range. In one case, treatment had been obviously inadequate (hemoglobin 49 per cent, red blood cell count 1.9 million). His serum iron level was 0.26 mg. per 100 c.c., dropping to within the normal range after one week of adequate treatment. The other case had the same serum iron level; treatment, previously adequate, had been discontinued. His blood count was not markedly abnormal (hemoglobin 97 per cent, red blood cell count 4.4 million).

In summarizing the observations in pernicious anemia we may note: untreated cases show uniformly high values, often slightly above the normal range;

one week of adequate liver therapy will bring the values down to within normal limits; lapsed or inadequate treatment will result in increased values, sometimes exceeding the normal limit.

*Serum Iron in Hypochromic Anemia.*—Animal experiments with horses<sup>27</sup> and with rabbits<sup>28</sup> have indicated that hemorrhage will result in depletion of serum or plasma iron. Barkan<sup>9</sup> has repeated some of these experiments and has shown that a decrease in plasma iron may be observed even when the hemoglobin and red blood cell count are not appreciably affected.

Riecker<sup>25</sup> investigated a number of clinical cases of secondary anemia and found low serum iron values. His values in general deviate so widely from the findings of other workers that his evidence is hardly admissible. Locke, Main, and Rosbash<sup>10</sup> report 2 cases, with serum iron values of 0.015 and 0.04 mg., respectively. Moore, Doan, and Arrowsmith<sup>18</sup> report 14 cases of hypochromic anemia, all but 3 with serum iron values below normal. Heilmeyer and Plötner<sup>21</sup> report 7 cases of acute anemia following hemorrhage, and 12 cases with chronic hemorrhage. The serum iron values fall between 0.01 and 0.04 mg. per 100 c.c.

Observations made at the Evans Memorial on patients with hypochromic anemia show that low serum iron values are the rule; values below the low normal limit (0.04 mg.) are uncommon. There is a consistent tendency for increase in serum iron values following successful treatment or cessation of blood loss. The findings in hypochromic anemia are summarized in Table II.

TABLE II

CASE	SERUM IRON MG. PER 100 C.C.	HEMOGLOBIN PER CENT
J. Hemophilia with rectal bleeding Treated with iron and transfusions (three months later)	0.03 0.23	26 91
T. Anemia associated with diabetes and hypertension After seven days' iron therapy	0.03 0.08	68 78
K. Nutritional anemia: chronic cholecystitis and multiple neuritis	0.03	74
R. Carcinoma of pharynx	0.03	61
F. Carcinoma of stomach	0.04	45
W. Duodenal ulcer, after hemorrhage One month later	0.04 0.15	68 104
H. Ulcerative colitis	0.06	58
B. Chronic nephritis	0.09	55
R. Pyonephrosis and nephrolithiasis	0.08	33
O. Toxemia of pregnancy After six weeks of iron therapy	0.05 0.08	62 75
C. Hematemesis One month later	0.13 0.22	58 84

*Serum Iron in Other Types of Anemia.*—Several cases studied did not permit easy classification, but showed such marked variations from the normal, as regards serum iron, that they deserve recording.

Mr. C., aged 83 years, was admitted November 24, 1936, and discharged December 19. He was followed as an outpatient for a time, was readmitted March 29, and died April 11, 1937. He had an anemia with achlorhydria, which improved slightly under liver therapy, but without reticulocyte response. He was followed for a time after his first discharge, then failed to appear for treatment. After about two months, he returned in much worse condition,

was readmitted, and died after a few days in the hospital. The anatomic diagnosis was "profound anemia (type undetermined in gross)." The bone marrow sections were examined by Dr. Charles F. Branch and by several other pathologists: the opinion was consistently given that there was a toxic depression of bone marrow activity, probably from infection. Although he apparently did not have pernicious anemia, the patient's serum iron figures are similar to those seen in pernicious anemia cases.

TABLE III  
SERUM IRON VALUES IN A CASE OF TOXIC DEPRESSION OF BONE MARROW

DATE	HEMOGLOBIN PER CENT	RED BLOOD CELL COUNT (MILLIONS)	SERUM IRON MG PER 100 C.C.
November 24	36	1.8	
November 30	52	2.1	1.16
December 7	42	2.0	0.42
January 9	49	2.0	0.21
January 19	44	1.9	0.23
January 26	42	2.0	0.19
March 30	24	1.2	0.25

It seems reasonable to believe that the reason for the high serum iron values is the same in this case as in pernicious anemia, adequate iron supply and storage with deficient utilization by the bone marrow. In both situations there is also the possibility of increased blood pigment destruction.

Mrs. G., aged 40 years, was admitted February 1 and discharged April 24, 1937, with a diagnosis of aplastic anemia, for which no definite cause was established. Her anemia was hyperchromic, with free hydrochloric acid present in the gastric contents. She received liver, iron, and several transfusions during her stay in the hospital, and was discharged not greatly improved. From the viewpoint of the behavior of the serum iron her case is somewhat similar to the preceding one. Feb. 3, 1.6 mg iron per 100 c.c. serum, Feb. 20, 0.35 mg; Feb. 25, 0.18 mg.; March 1, 0.31 mg.; April 1, 0.27 mg.

Mr. F., aged 54 years, was admitted September 24, 1936. He had a hyperchromic anemia, reticulocytosis, achlorhydria, enlarged liver and spleen, and serologic evidence of syphilis. He was deeply jaundiced. Treatment with iron, liver, and transfusions did not lead to any great improvement. He was discharged November 18, with diagnoses of tertiary syphilis and acquired hemolytic jaundice. His hemoglobin varied between 24 and 32 per cent, his red blood cell count from 0.9 to 1.2 million. Serum iron determinations: September 24, 0.64 mg.; October 7, 0.28 mg.; October 16, 0.21 mg.; October 26, 0.23 mg.; October 29, 0.41 mg.; November 9, 0.76 mg.; November 15, 0.54 mg. The excessive iron in his serum might reasonably be attributed to his accelerated rate of blood pigment destruction.

*Serum Iron in Erythremia*—Two cases of untreated polycythemia vera were reported by Moore, Doan, and Arrowsmith,<sup>18</sup> both showing low normal levels. We have had opportunity to study two cases; in one case our findings were similar to those cited; in the other, values slightly above normal range (0.35 to 0.26 mg.) were obtained on repeated examination. The available observations are quite obviously inadequate to make any attempt at conclusions.

#### SUMMARY

1. A simple method for the measurement of the total iron of blood serum is described.

2. The normal range of iron in nonhemolyzed blood serum is from 0.04 to 0.23 mg. per 100 c.c. serum. The average amount is about 0.13 mg.

3. In pernicious anemia the blood serum iron is moderately increased, and returns to normal with successful liver therapy.

4. In hypochromic anemias the values for serum iron are low, but not necessarily below the normal range. Iron therapy increases the serum iron level in these cases.

5. In aplastic and hemolytic anemias the serum iron levels may be elevated.

#### REFERENCES

1. Sachs, A., Levine, V. E., and Appelsis, A.: Iron in Human Blood, *Arch. Int. Med.* 52: 366, 1933.
2. Helmer, O. M., and Emerson, C. P.: The Iron Content of the Whole Blood of Normal Individuals, *J. Biol. Chem.* 104: 157, 1934.
3. Haden, R. L.: The Determination of Hemoglobin by the Iron Content Method, *J. Lab. & Clin. Med.* 19: 406, 1934.
4. Johnson, M., and Hanke, M. E.: The Iron Content and Oxygen Capacity of Blood, *J. Biol. Chem.* 114: 157, 1936.
5. Biernacki, E.: Untersuchungen über die chemische Blutbeschaffenheit bei pathologischen, insbesondere bei anämischen Zuständen, *Ztschr. f. klin. Med.* 24: 460, 1894.
6. Erben, F.: Die chemische Zusammensetzung des Blutes bei perniziöser Anämie, *Ztschr. f. klin. Med.* 40: 267, 1900.
7. Fowell, P. H. C.: Iron in the Blood, *Quart. J. Med.* 6: 179, 1912.
8. Butterfield, E. E.: Über die Lichtextinktion, das Gasbindungsvermögen und den Eisengehalt des menschlichen Blutfarbstoffs in normalen und krankhaften Zuständen, *Ztschr. f. physiol. Chem.* 62: 173, 1909.
9. Barkan, G.: a. Zur Frage der Einwirkung von Verdauungsfermenten auf das Hämoglobineisen, *Ztschr. f. physiol. Chem.* 148: 124, 1925.  
b. Über das leicht abspaltbare Bluteisen und sein Verhältnis zum Hämoglobin, *Ibid.* 171: 179, 1927.
- c. Die Verteilung des leicht abspaltbaren Eisens zwischen Blutkörperchen und Plasma und sein Verhalten unter experimentellen Bedingungen, *Ibid.* 171: 194, 1927.
- d. Über Säurelösliches Eisen im Blutserum, *Klin. Wehnschr.* 6: 1615, 1927.
- e. Über den Gehalt des Blutserums am Eisen, *Ztschr. f. physiol. Chem.* 177: 205, 1928.
- f. (and Berger, E.) Differenzierung des leicht abspaltbaren Bluteisens auf Grund seiner Reaktion mit Kohlenoxyd und Sauerstoff, *Arch. f. exper. Path. u. Pharmacol.* 136: 278, 1928.
- g. Zur Frage des Blutfarbstoffzerfalls durch verdünnte Säuren, *Biochem. Ztschr.* 224: 53, 1930.
- h. Über Bestimmungsmethodik und Eigenschaften des "leicht abspaltbaren," Bluteisens, *Ztschr. f. physiol. Chem.* 216: 1, 1933.
- i. Über das Verhalten von anorganischem Eisen nach Zusatz zum Blute, *Ibid.* 216: 17, 1933.
- j. Die Unterscheidung des "leicht abspaltbaren" Bluteisens vom Hämoglobineisen und vom anorganischen Eisen, *Ibid.* 221: 241, 1933.
- k. Neuere Untersuchungen über das Bluteisen, *Forsch. u. Fortschr.* 10: 114, 1934.
- l. Verfahren zur Bestimmung des leicht abspaltbaren Bluteisens, *Handb. d. biol. Arbeitsmeth.* (E. Abderhalden, editor), Abt. V, Teil 8, Seite 1207, 1935.
- m. (with Olesk, J.) Zur Frage der Identität der Blutkatalase mit dem leicht abspaltbaren Bluteisen, *Ztschr. f. physiol. Chem.* 236: 197, 1935.
- n. Weitere Untersuchungen über das säurelösliche Plasmaeisen, *Ibid.* 239: 97, 1936.
- o. (and Sehales, O.) Trennung der Blutkatalase vom "leicht abspaltbaren" Eisen, *Ibid.* 244: 81, 1936.
- p. (and Sehales, O.) Hämoglobin und "leicht abspaltbares" Eisen bei der Adsorption und bei der Kataphorese, *Ibid.* 244: 257, 1936.
- q. Das "leicht abspaltbare" Bluteisen und das Serum Eisen, *Klin. Wehnschr.* 15: 169, 1936.
- r. Vereinfachtes Verfahren zur Bestimmung des "leicht abspaltbaren" Bluteisens, besonders des Serum- bzw. Plasmaeisens, *Ibid.* 16: 300, 1937.
- s. Chemischer Aufbau und physiologische Bedeutung des "leicht abspaltbaren" Bluteisens, *Ztschr. f. physiol. Chem.* 248: 96, 1937.
10. Locke, E., Main, E. R., and Rosbash, D. O.: The Copper and Nonhemoglobinous Iron Contents of the Blood Serum in Disease, *J. Clin. Investigation* 11: 527, 1932.
11. Warburg, O., and Krebs, H. A.: Über locker gebundenes Kupfer und Eisen im Blutserum, *Biochem. Ztschr.* 190: 143, 1927.
12. Klumpp, T. G.: The Variability of Non-hemoglobin Iron, *J. Clin. Investigation* 14: 351, 1935.

- 13 Josephs, H W The Iron of the Blood Bull Johns Hopkins Hosp 50 50, 1935
- 14 Josephs, H W, and Winocur, P The Occurrence of Nonhemoglobin Iron in Whole Blood, *Ibid* 61 75, 1937
- 15 Moore, C V Chemical Methods and Normal Values for Plasma Iron and "Easily Split Off" Blood Iron, *J Clin Investigation* 16 613 1937
- 16 Fowweather, F S The Determination of Iron in Blood Plasma *Biochem J* 28 1160, 1934
- 17 Tompsett, S L Thiolacetic Acid as a Reagent for the Determination of the Inorganic Iron Content of Certain Biological Materials *Ibid* 28 1536 1934
- 18 Moore, C V, Don, C A, and Arrowsmith W R The Mechanism of Iron Translocation Its Significance in Iron Utilization in Anemic States of Varied Etiology, *J Clin Investigation* 16 627, 1937
- 19 Bing, F C, and Hanzli, R F A Method for the Estimation of Serum Iron, *Proc Soc Exper Biol & Med* 32 1013 1935
- 20 Bing, F C Purification of Benzidine and an Improved Reagent, for Estimating Hemoglobin in Blood, *J Biol Chem* 95 387 1932
- 21 Wong, S Y Colorimetric Determination of Iron and Hemoglobin in Blood, *Ibid* 77 409, 1928
- 22 Evelyn, K A Personal communication
- 23 Guthmann, H, Bruckner, M A, Ehrenstein and Wagner H Das ultrafiltrable Eisen im Serum der Frau, *Arch f Gynak* 147 469 1931
- 24 Starkenstein, F, and Weden, H Über das anorganische Eisen des Organismus *Arch f exper Path u Pharmacol* 134 274 1928
- 25 Riecher, H H Iron Metabolism in Pernicious and in Secondary Anemia, *Arch Int Med* 46 458, 1930
- 26 Marlow, A, and Taylor, F H L Constancy of Iron in the Blood Plasma and Urine in Health and in Anemia, *Arch Int Med* 53 301 1934
- 27 Fontes, G, and Thivolle, L Non hemoglobin Iron content of Serum and Its Diminution in Experimental Anemia, *Compt rend Soc de biol* 93 687 1925
- 28 Thoenes, F, and Aschaffenburg R Der Eisenstoffwechsel des wachsenden Organismus, Berlin, 1934, S Karger (Quoted from Barkan)
- 29 Jenkins, C E, and Thompson, M L The Distribution of Iron in Blood, *Brit J Exper Path* 18 175, 1937
- 30 Heilmeyer, L, and Plotner, K Das Serum-eisen und die Eisenmangelkrankheit, Jena, 1937, Gustav Fischer

## MODIFIED APPARATUS FOR OBTAINING GASTRIC CONTENTS\*

JULES L POSNER, B S, JOHN T MYERS, PH D, M D, AND ANDREW R FODOR,  
B A, NEW YORK, N Y

SINCE 1812, when it was first used by Philip S Physick, and in 1822, by Jukes, as a means of relieving the stomach of poisons, the stomach tube has undergone little modification. In 1869, Kussmaul used it clinically for pyloric carcinoma and spasm, first experimenting on a professional sword swallower.

The general structure of the tube has been functionally preserved. Its total length is about 38 inches, of which about 20 to 24 inches extend from the mouth to the stomach. This allows 5 to 8 inches to dip into the stomach. The diameter of the older type tube (Boas) is approximately 12 mm. The distal end tapers to an opening and has two or more smaller openings in the side of the tube,  $\frac{1}{2}$  to 1 inch from the tip.

The tube is introduced into the pharynx, and the patient is instructed to close his lips around it and breathe rapidly in order to avoid undue abdominal

\*From the Research Department of Bendiner and Schlesinger Laboratories New York  
Received for publication March 28 1938



stress. The end of the tube is suspended in a flask in which the stomach contents is collected. Cummer, as well as Todd and Sanford, has suggested that a Pollitzer bag be attached to the end of the tube, which is compressed until the esophagus is entered. Release of the bag then causes suction, forcing the stomach contents into the bag.

This process is rather awkward. A single compression is not always sufficient to free a badly clogged tube. The bag must then be removed, recompressed, and attached to the tube as often as necessary to obtain sufficient material. Direct recompression without removal would force the material back through the tube. The bag must be carefully cleaned and sterilized before each case.

The duodenal tube was devised in 1895 by J. C. Hemmeter and later modified by Kuhn, Einhorn, Rehfuss, and others.

The Rehfuss modification is most extensively used both for gastric and duodenal extractions. A small olive-shaped metal tip terminates this tube. Due to its small diameter, 3 to 4 mm., the patient will bear it comfortably, but it becomes easily clogged, making it essential to use suction. This is directly applied with a large aspirating syringe attached to the tube. The difficulties of this arrangement are obviously similar to those encountered in the use of the Boas tube. In spite of any precautions, the authors have found that mucus and food particles will often clog the tube, necessitating its removal for washing and causing considerable discomfort to the patient. We have attempted to introduce distilled water into the clogged tube to produce syphon action, but results have been too variable.

#### PROCEDURE

The modified procedure which the authors submit for approval may be used with the Boas or Rehfuss tubes, either for fractional or occasional extraction of gastric contents. This is a modified suction method with the addition of a Florence type flask to serve both as a suction generator and a trap for the extracted material. The flask is tightly fitted with a two-holed rubber stopper. Through one opening is passed a hard rubber nozzle extending slightly into the flask. A piece of hard glass of adequate diameter and of sufficient length to reach from 1 inch above the stopper to about halfway above the bottom of the flask is passed through the other hole.

The end of the gastric tube is attached to the upper end of the glass tube. A rubber bulb with a hard rubber opening to fit tightly over the protruding nozzle is obtained. The apparatus sold by the Davol Rubber Co., as the "Ewald Evacuator," with a rubber bulb of 12 ounces capacity and a glass window in the tube by means of which the flow of material may be observed, has been most effectively used by the authors. This outfit is easily adaptable for this procedure by manipulating as suggested above. The tube is introduced into the stomach, the bulb is compressed, placed on the nozzle of the receiving flask and released. This causes the formation of a slight vacuum, which is usually sufficient to start the flow of gastric fluid into the receiving flask. Food particles or mucus which now clog the tube are freed by slight compression and release

of the bulb which remains fixed on the nozzle. The bulb never comes in direct contact with the extracted material, so that ordinary cleaning and drying is sufficient to prepare it for use (Fig 1)

When fractional extraction is required, a pinch clamp may be used to close the tube when a sufficient amount of sample has been collected.

The described method has been used by the authors on patients suffering from a variety of disorders, from normal to advanced cases of carcinoma. Particularly successful results have been obtained in aspirating gastric contents from a fasting stomach for bacteriologic examination especially in connection with the newer technique for diagnosis of pulmonary tuberculosis. We have yet to experience failure in obtaining sufficient substance for examination or to note

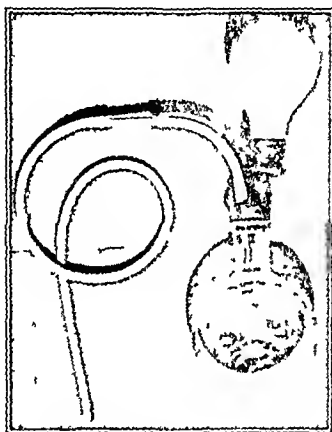


Fig 1—Photograph of described apparatus

any harmful effects of the procedure on the patient. On the contrary, the patient suffers less discomfort than in any of the current procedures, due greatly to the speed with which the whole operation can be accomplished.

#### SUMMARY

An improved method for the removal of gastric contents having the following advantages is described:

- 1 Clogging of the tube and repeated washings are made unnecessary because of indirect siphon action.
- 2 Larger quantities of test meals are recovered, permitting more accurate analyses and interpretation.
- 3 It permits the removal of gastric material without previous ingestion of test meals.
- 4 It decreases the discomfort of the patient due to greater speed and certainty of action.

## REFERENCES

1. Cummer, C. L.: *Manual of Clinical Laboratory Methods*, Philadelphia, 1926, Lea & Febiger, p. 319.
2. Hillsman, J. A.: *Continuous Gastric Lavage*, *Canad. M. A. J.* 29: 186, 1933.
3. Physick, P. S.: *Account of a New Mode of Extracting Poisonous Substances From the Stomach*, *Eclectic Repertory*, Philadelphia 3: 111, 1812-13. *Ibid.* 3: 380. After submitting this paper for publication it was found that Dr. Physick had later on given credit for the invention to Dr. Alexander Monro of Edinburgh who had published it in his *Inaugural Thesis* in 1797. This was unknown to Dr. Physick at the time of his original article.
4. Jukes, E.: *A Method of Saving Life in Cases of Poisoning, by Means of a Pump*, *Med. Repertory*, London 2: 112, 1822.
5. Kussmaul, A.: *Ueber die Behandlung der Magenweiterung durch eine neue Methode*. *Deutsches Arch. f. klin. Med.* 6: 385, 1895.
6. Hemmeter, J. C.: *An Apparatus for Washing Out the Stomach and Sigmoid*, *New York M. J.* 61: 385, 1895.

## THE DETERMINATION OF MORPHINE IN THE URINE OF MORPHINE ADDICTS\*

FRED W. OBERST, M.S., PH.D., LEXINGTON, KY.

A QUANTITATIVE determination of morphine in urine consists of the isolation of the alkaloid in a relatively pure state and the measurement of its concentration. Most of the methods reported in the literature are satisfactory only when the amounts of morphine in urine are relatively high as in cases of acute morphine poisoning or in experimental animals receiving a high morphine intake. The amount of morphine found in the urine of clinical cases and of most morphine addicts is considerably lower, consequently the methods described are not practical, since it becomes necessary to use excessively large volumes of urine to carry out an analysis. Because the question of the fate of morphine is being studied in the addict, it became necessary to evaluate the various methods already described and to develop a more practical and sensitive method. The procedure of Pierce and Plant<sup>1</sup> still is the most satisfactory quantitative method for the determination of morphine in biologic materials. Their extraction procedure for a quantitative removal of morphine from urine has not been surpassed. They removed morphine with an ethyl alcohol-chloroform mixture from 60-70 ml. of urine saturated with sodium bicarbonate using a continuous liquid-liquid extractor. The residue obtained after the evaporation of the solvent was dissolved in 0.5 per cent hydrochloric acid and subjected to three extractions with an amyl alcohol-chloroform mixture to remove impurities. Then they saturated the aqueous solution with powdered sodium bicarbonate and removed the alkaloid by three extractions with an amyl alcohol-chloroform mixture. The extraction solvent containing the morphine was treated with a few drops of dilute hydrochloric acid and evaporated to dryness. The morphine concentration in the final residue, ranging from 1 to 20 mg., was determined

\*From the United States Public Health Service Hospital, Lexington.

Read before the Federation of American Societies for Experimental Biology, Baltimore, Md., March 30—April 2, 1938.

colorimetrically by means of the Lautenschlager diazobenzenesulphonic acid method.<sup>2</sup> Then studies were made on dogs receiving doses of morphine ranging from 10 to 50 mg per kg body weight, consequently large amounts of the drug were excreted in the urine.

Many other methods for the isolation and determination of morphine are described in the literature, most of the earlier methods are adequately reviewed by Balls and Wolff<sup>3</sup> and Decker.<sup>4</sup> Most of these methods are strictly qualitative. Balls and Wolff,<sup>3</sup> Fry, Light Torrance and Wolff<sup>5</sup> and Wolff, Riegel, and Fry<sup>6</sup> used a continuous liquid liquid extractor and made repeated isoelectric extractions until the residue was pure enough for a silicotungstic acid precipitation. The precipitate was removed, dried and weighed. This method lacked sensitiveness, at least 5 mg and better 10 mg of morphine being required for a reasonably accurate analysis. When the amount of morphine in a single determination was less than 8 mg, the final estimation was made colorimetrically by the diazo reaction.

Loofs<sup>7</sup> in 1922 was one of the first to study successfully the excretion of morphine clinically in human beings. He removed morphine from urine by the classical Stas Otto procedure of repeated evaporations and acid alkaline extractions. The final residue was tested by one of the well known alkaloidal color reagents (Froehde, Marquis, Pellagri). This method required the use of large quantities of urine and detected only the presence of morphine.

Panse<sup>8</sup> tried to simplify the Stas Otto method and limited the quantity of urine for an analysis to 50-100 ml. He used ethyl acetate to extract the morphine and then sublimed the alkaloid for its detection by the usual alkaloidal color reagents. Teruuchi and Kai,<sup>9</sup> Ellinger and Seeger,<sup>10</sup> To and Rin,<sup>11</sup> Frohden and Huang,<sup>12</sup> Liang<sup>13</sup> and others reported various procedures for extracting morphine from urine. The presence of morphine in the final residue was determined by the usual alkaloidal color or precipitation tests. Teruuchi and Kai determined the concentration by titration according to Gordin's method.

Decker<sup>4</sup> described a method suitable for clinical use in which morphine was extracted from urine by means of ethyl acetate and was determined nephelometrically as a morphine molybdate vanadate complex, the turbidity produced being roughly proportional to the amount of morphine present. He claimed that his method is rapid and sensitive, and that he obtained a satisfactory morphine test on 10 ml urine containing as low as 0.01 mg morphine.

With the exception of the Decker method it is apparent that the methods for the quantitative determination of morphine in a single extraction from urine are not satisfactory for amounts below 1 mg. In the following report methods and procedures are presented for the quantitative determination of 0.03 to 2.0 mg of morphine in 10 to 70 ml urine. A new method is described for the further separation of morphine from impurities in a urinary residue by means of permittit. The morphine concentration in the permittit is then determined colorimetrically by a new procedure for morphine, which is more sensitive than previous colorimetric methods. The Decker method has been improved and made more reliable for the detection and determination of small amounts of morphine.

## EXPERIMENTAL

*Reagents.*—Sodium bicarbonate, U.S.P., powdered.

Ethyl alcohol, 95 per cent, U.S.P., is treated with charcoal (Norit), filtered, redistilled on a water bath, leaving about 10 per cent in the distilling flask.

Chloroform, U.S.P., is washed with a small volume of dilute sodium bisulfite, and after redistilling twice on a water bath at 80° C., is stored in dark amber bottles.

Amyl alcohol is prepared by washing fusel oil or amyl alcohol, Tech., four times with equal volumes of water, finally discarding the wash water. The remaining solution is distilled on a hot plate, collecting the fraction boiling between 125° and 133° C. After redistilling a second time to increase its purity, it is stored in dark amber bottles.

Hydrochloric acid (0.5 per cent, 50 ml. of concentrated acid diluted with water to 2,000 ml.).

Permuit, according to Professor Otto Folin.

Phenol reagent, Folin and Denis.<sup>14</sup> This reagent is prepared as follows: 100 gm. sodium tungstate dissolved in 750 ml. water, 20 gm. phosphomolybdic acid, and 50 ml. 85 per cent phosphoric acid. The mixture is boiled for two hours, and after cooling is diluted to one liter.

Sodium Carbonate, C.P. (saturated solution).

The diazo mixture is prepared by mixing equal volumes of aqueous solutions of sodium sulphanilate (4 per cent), of hydrochloric acid (6 ml. concentrated acid, specific gravity 1.18 in enough water to make 100 ml.), and of sodium nitrite (1.4 per cent). The reagent should be freshly mixed and allowed to stand for five minutes before using.

Sodium hydroxide (10 per cent).

Ammonium molybdate, C.P. (10 per cent).

Sulfuric acid (2.5 Normal).

Ammonium vanadate, C.P. (2 per cent and filter).

A morphine standard containing 1 mg. of base per ml. solution is prepared by dissolving 0.3325 gm. purified morphine sulfate ( $C_{17}H_{19}O_5N$ ),  $H_2SO_4 + 5H_2O$  in 250 ml. water.

*Extraction Procedure.*—The quantitative removal of morphine from urine was carried out by the Pierce and Plant<sup>1</sup> procedure for dog urine. The procedure reported by them is as follows:

“Sixty cubic centimeters of fresh dog’s urine are saturated with powdered sodium bicarbonate, giving a distinctly alkaline reaction to litmus. The alkaline urine is transferred to a liquid-liquid extractor in which 110 to 125 c.c. of a mixture of one part ethyl alcohol (95 per cent) and three parts chloroform has been placed, one-fourth the amount being in the extraction chamber, the rest in the extractor flask along with 0.5 c.c. of concentrated hydrochloric acid. Enough heat is applied to keep the solvent in active ebullition, and extraction continued for ninety to one hundred and twenty minutes. This is equivalent to treating the alkaline urine with 10 to 20 volumes of the solvent, and removes all of the morphine along with the small amounts of other substances. The alcohol-chloroform extract is transferred to an evaporating dish, together with several small portions of 0.5 per cent aqueous hydrochloric acid used to rinse the flask, and evaporated to dryness on a water bath.

The residue is then treated, first with 10 c.c., then twice with 5 c.c. of a 0.5 per cent hydrochloric acid, and the dish rinsed with two or three small portions of distilled water; the combined extracts and washings, usually about 35 c.c., are transferred without filtration to a 125 c.c. cylindrical separatory funnel and extracted three times with an equal volume of a mixture of one part amyl alcohol and three parts chloroform. During each of the extractions the funnel is shaken for five minutes in a mechanical shaker. The organic solvent is discarded and the aqueous solution saturated, in the funnel, with powdered sodium bicarbonate. The aqueous alkaline solution is then extracted three or four times with one and a half to two volumes of the amyl alcohol-chloroform mixture, shaking for five to ten minutes in each extraction. The combined amyl alcohol-chloroform extracts are then acidulated with dilute hydrochloric acid and evaporated to dryness on a water bath. Prolonged heating

of the dry residue from this or from the ethyl alcohol-chloroform extract should be avoided. The residue is then dissolved in a suitable amount of 0.5 per cent hydrochloric acid and the amount of morphine in it determined."

Fig 1 is a photograph of the continuous liquid liquid extractor having a maximum capacity of 70 ml of urine of the form used by Balls and Wolf and modified by Pierce and Plant. For certain experimental studies we have used a larger liquid liquid extractor, the extraction column of which was filled with glass beads to increase the surface area exposed to the extracting liquid. This

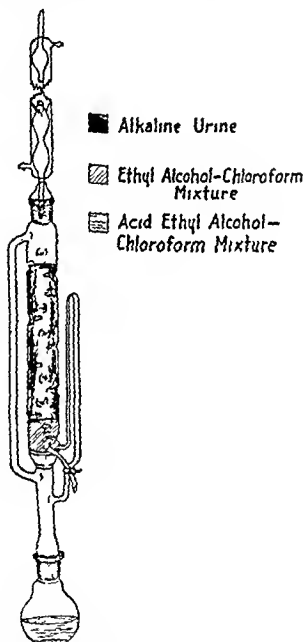


Fig 1—Continuous liquid liquid extractor

extractor so used has a maximum capacity of 150 ml and separates satisfactorily very small amounts of morphine to be determined by the improved Decker method. We have fitted these extractors with the interchangeable ground glass connections, made by Ace Glass Inc, which are especially desirable when dealing with small amounts of morphine in urine. Unless special high grade corks are available, considerable amounts of impurities are extracted from them. They also must be changed frequently, for the extraction reagents readily decompose them. Rubber stoppers are not very satisfactory, for they swell considerably after several extractions.

*The Separation of Morphine From a Urinary Extract by Means of Permutit.*—Four grams of permutit are placed in a 50 ml. volumetric flask and washed twice with distilled water, the supernatant fluid being discarded. The urinary residue obtained after the evaporation of the organic solvent is dissolved in 2 or 3 ml. of water and is transferred quantitatively by several washings to this flask. If insoluble matter is present after dissolving the residue with water, it must first be removed by filtration, the small filter paper being thoroughly washed four or five times and all wash water added to the permutit. This solution is frequently shaken several minutes with the permutit for about half an hour to allow the morphine to react with the permutit. After the permutit has settled to the bottom of the flask, the supernatant fluid is withdrawn by suction through a drawn tube or by decantation. The permutit is washed three times with 10-15 ml. portions of distilled water to remove all soluble impurities. The morphine concentration in the permutit is determined by any suitable colorimetric procedure.

Great care must be taken not to wash out any permutit from the flask. Flat bottom flasks, such as volumetric flasks, are much more suitable than graduated test tubes for the extraction of morphine by permutit, since they allow a larger surface area of permutit to be exposed to the liquid phase.

The dissolved urinary residue must be neutral when it is shaken with permutit. If the solution should be slightly too alkaline or too acidic, morphine will not be removed quantitatively by means of the permutit. Those bases whose "strength" is represented by a dissociation constant of  $5 \times 10^{-9}$ , or greater, are removed by permutit.<sup>15</sup> Ammonia reacts with permutit but does not give a color with any of the morphine color reagents, consequently enough permutit must be present to combine with both. Ordinarily 4 gm. of permutit are sufficient to combine with morphine in a residue up to a concentration of about 1.5 mg. per 65 ml. of urine. One gram of permutit will completely remove 1.0 mg. of morphine from a water solution.

The complete removal of morphine with permutit from a solution of urinary residue extracted by the regular procedure was tested by transferring the solution, after shaking with permutit, to a second flask containing permutit. The solution was again shaken with permutit, after which it was discarded. The permutit in each flask was washed three times with water and finally treated by the reagents used in the new colorimetric method. Color developed in the first flask as usual, but was absent in the second. A number of such tests indicated the complete removal of morphine in the residue by permutit in amounts up to about 1.5 mg.

*A Colorimetric Method for the Determination of Morphine Using a Phenol Reagent.*—The removal of morphine from fresh urine is carried out by the Pierce and Plant procedure, except that the acidulated residue from the liquid-liquid extractor is treated only once with the amyl alcohol-chloroform mixture. After treating the solution of urinary extract with permutit in the 50 ml. volumetric flask, the morphine concentration in the permutit is determined colorimetrically by the addition of 6 ml. saturated sodium carbonate and 2 ml. of Folin-Denis phenol reagent.<sup>14</sup> A blue color develops immediately which is quite stable for a day. The standards are prepared by the addition of known

amounts of morphine to 4 gm of washed permutit in similar flasks, followed by treatment with sodium carbonate and phenol reagent. The flasks are filled to volume with distilled water, the contents thoroughly mixed and, after two or three hours, the unknowns are compared with the standard in a colorimeter. The use of a No RG1 filter (red), made by Jena Glass Works, placed in the eye piece of the colorimeter greatly facilitates the ease in matching the colors, especially if the blue color is very weak.

This color test is very sensitive for small amounts of morphine. Amounts as low as 0.05 mg, diluted to 50 ml, can be read in a colorimeter fitted with the No RG1 filter. Smaller amounts of morphine in a correspondingly reduced volume can be determined, provided it is certain that the solution is practically free of other reducing substances.

Uric acid and phenols must be completely removed from a urinary residue, for they give a blue color with the phenol reagents. Since these substances do not react with permutit, they can be removed by washing.

TABLE I

## RECOVERY OF MORPHINE ADDED TO URINE

The recovery of morphine added to urine using the liquid liquid extraction process followed by further purification with acid and alkali extractions. The color was developed by the addition of sodium carbonate and phenol reagent to the permutit.

URINE VOLUME	MORPHINE ADDED TO URINE	MORPHINE RECOVERED	EXPERIMENTAL FFPOR
ml	mg	mg	mg
50	0.00	0.044	0.024
70	0.05	0.077	0.025
50	0.05	0.041	0.05
50	0.10	0.108	0.008
50	0.10	0.103	0.003
75*	0.00	0.240	—
75*	0.10	0.343	0.003
50	0.20	0.200	0.000
50	0.20	0.200	0.000
50	0.20	0.180	0.20
50	0.20	0.174	0.26
50	0.0	0.100	0.000
50	0.40	0.311	0.49
50	0.40	0.364	0.37
70	0.50	0.484	0.12
70†	0.50	0.450	0.50
70	0.70	0.640	0.4
65	0.80	0.079	-0.01
70	1.00	0.990	-0.10
70	1.50	1.530	0.630
50	2.00	1.980	0.20

\*Urine from a morphine addict

†Water solution extracted in liquid liquid extractor

‡Trace of color due to extracted impurities equivalent to 0.024 mg morphine

Morphine free urinary extracts obtained from 65 ml urine treated with permutit, followed by the addition of sodium carbonate and phenol reagent, gave a color in the final solution which, when compared with standards, was equivalent to 0.0205 mg morphine. Small quantities of morphine are unavoidably lost in the extraction process. This loss is nearly compensated by the small amount of color obtained from impurities carried over from the urine. Table I shows that varying amounts of morphine added to urine have been satis-



factorily recovered by this method. Table II is presented to show the concentration of morphine in the urine of two uncontrolled addicts. It is not intended that any conclusions concerning morphine excretion be drawn from this table. A detailed study on morphine excretion is in progress.

TABLE II

## MORPHINE CONTENT OF URINE FROM MORPHINE ADDICTS

Addict 1. Intake 160 mg. morphine sulfate (120.3 mg. base) per day

DATE	URINE VOLUME	MORPHINE BASE IN 70 ML. URINE	MORPHINE BASE PRESENT IN 24- HOUR-URINE SPEC- IMEN	PER CENT MORPHINE BASE EXCRETED IN URINE
	<i>ml.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1/ 7/38	1425	0.320	6.25	5.19
1/ 8/38	1350	0.450	8.69	7.21
1/ 9/38	1375	0.279	5.48	4.55
1/10/38	825	0.685	8.07	6.70
1/11/38	800	0.953	10.90	9.06
1/12/38	950	0.408	5.54	4.60
1/13/38	1450	0.377	7.80	6.48

Addict 2. Intake 200 mg. morphine sulfate (150.4 mg. base) per day

1/ 6/38	1600	0.375	8.58	5.70
1/ 7/38	1950	0.395	11.00	7.31
1/ 8/38	1350	0.500	9.65	6.42
1/ 9/38	1700	0.368	8.92	5.93
1/10/38	2050	0.321	9.40	6.25

*Diazotization Method for the Determination of Morphine.*—The diazo reaction used in the determination of 1-10 mg. morphine in a urinary residue prepared by the Pierce and Plant procedure is carried out by dissolving the residue in 2 ml. of water followed by 1 ml. of diazo mixture and 2 ml. 10 per cent sodium hydroxide. After standing one and one-half hours, the solution is diluted to 10 ml. and the color compared in a colorimeter with a standard morphine solution treated in a similar manner. One milligram of morphine is about the minimum quantity which gives a satisfactory color by the diazo reaction when diluted to a final volume of 10 ml.

A residue obtained from 50 ml. morphine-free urine treated by the above diazo reaction gave a similar red color which was too dark for a morphine-free urine. The color intensity was not constant for different morphine-free urines. The recovery of 1.0 mg. morphine added to 50 ml. of urine and extracted by the usual manner gave in one instance a value of 1.5 mg. when compared with a 1.0 mg. standard. Apparently there are present substances other than morphine in the urinary residue which give a color reaction with the diazo reagents.

An experiment was made on a solution of urinary extract in which a large portion of the interfering substances giving the diazo color reaction was removed from the morphine by means of permutit. After washing the permutit three times with water, the diazo color was developed by the addition of the diazo mixture and the 10 per cent sodium hydroxide. The entire contents were heated for fifteen minutes on a boiling water bath. The color obtained was much less than that from a similar residue not treated with permutit.

The presence of gas bubbles suspended in solution which collected on the walls of the colorimeter cups and prisms was frequently observed while making readings for morphine in urine. To determine whether or not the presence of these bubbles would cause significant variations, an experiment was carried out using a set of three 10 mg morphine standards. After the addition of the diazo mixture and standing one and one half hours, the colors did not match satisfactorily with each other. Another set of three standards prepared similarly, but without the preliminary one and one half hours' standing, was heated fifteen minutes in boiling water. After cooling and adjusting the volume to 10 ml, the colors were compared with each other. They matched exactly with each other, but were nearly twice as strong as the unheated ones. No gas bubbles suspended in the solution were present. After the two sets of standards were allowed to stand for twenty four hours at room temperature, they were again compared with each other. The color of the unheated standards compared exactly with each other and had become darker, nearly approaching those of the heated ones. The size and concentration of these bubbles appeared to vary considerably with time and temperature, thus causing variable readings on the same sample. In the absence of these bubbles in both the standard and unknown, reproducible colorimetric readings were obtained. The heating process nearly doubled the sensitivity of the method and cleared the solution of suspended gas bubbles which interfered with the colorimetric readings.

*The Improved Decolor Method—A Qualitative Procedure* The presence of morphine in the urine of morphine addicts may rapidly be determined by making two extractions with ethyl acetate followed by the formation of a morphine molybdate vanadate complex. Approximately 35 ml of fresh urine is made alkaline by saturating it with USP sodium bicarbonate. After filtering, 25 ml of the filtrate is measured into a 150 ml separatory funnel. To this is added an equal volume of ethyl acetate. The two solutions are vigorously shaken for two or three minutes and then allowed to separate. The lower layer which is urine, is drawn off into a beaker for a repeated extraction. Rotating the separatory funnel back and forth a few times causes small amounts of urine adhered to the walls of the funnel to settle to the bottom for removal from the ethyl acetate. Since it is not possible to remove all traces of urine from the separatory funnel by draining, it is best to remove the ethyl acetate solution by pouring it carefully from the top of the separatory funnel into a dry filter paper and collect the filtered ethyl acetate in an evaporating dish. Traces of urine carried over with the ethyl acetate solution are very objectionable and must be kept to a minimum. The separated urine is treated once again with ethyl acetate, which, when separated from the urine, is added to the first portion with about 0.2 ml of 0.5 per cent hydrochloric acid and the entire volume evaporated to dryness.

The residue is dissolved in 15 ml of distilled water and 0.2 ml of 2.5 N sulfuric acid. To this is added 0.2 ml of 10 per cent ammonium molybdate, which on standing about ten to twenty minutes, precipitates most of the impurities from the solution but does not remove an appreciable amount of morphine. The precipitate is removed by filtering the fluid through Whatman No. 42 filter paper, about 30 mm in diameter, into a graduated cylinder.

The evaporating dish and filter paper are washed with three 0.5 ml. portions of distilled water, the washings being added to the first filtrate. The total volume should not exceed 3.0 or 3.2 ml. Two drops (0.1 ml.) of 2 per cent filtered ammonium vanadate are thoroughly mixed with this clear filtrate. After standing for a short period of time, a turbidity sets which appears as a white, finely divided, granular substance, remaining in suspension for a long time. The degree of this turbidity is roughly proportional to the amount of morphine present. In the absence of morphine there may be a slight precipitate in suspension, which is clear and translucent, but when examined by direct light at an oblique angle does not appear white and finely divided. Amounts of morphine as low as 0.03 mg. in 25 ml. of urine can readily be detected.

Occasionally a urine will form an emulsion with ethyl acetate which does not separate readily even after long standing. The emulsion can best be broken up by filtering it through a dry filter paper. If not all of it passes through the filter, the remaining portion is transferred to another dry filter paper. Ethyl acetate will not pass readily through a wet filter paper.

The original Deckert method was modified, after a number of attempts to detect the presence of small amounts of morphine had failed. When larger amounts of the drug were present, a positive result was obtained. Studies were then carried out to determine the factors responsible for changes in the sensitivity of the method. In addition to morphine large quantities of impurities are extracted by the ethyl acetate which are precipitated by ammonium molybdate and ammonium vanadate. These impurities must be separated from morphine as well as possible to prevent them from masking the morphine reaction. This separation was accomplished by precipitation of the impurities with ammonium molybdate at the isoelectric point followed by filtration. Morphine molybdate is not precipitated in concentrations ordinarily found in these experiments nor is an appreciable amount lost with the precipitate by absorption. Nitric, hydrochloric, and sulfuric acids were tested in the separation of these impurities and for the formation of the morphine-molybdate-vanadate complex. The best results were obtained by dissolving the urinary residue in 1.5 ml. water, followed by the addition of 0.2 ml. 2.5 N sulfuric acid. After adding ammonium molybdate, filtering, and washing the filter paper, there must be a definite acid concentration in the total filtrate to obtain the maximum separation of the morphine-molybdate-vanadate complex by the addition of ammonium vanadate. Excess acid in the solution increases the solubility of the complex. It appeared that with sulfuric acid the morphine-molybdate-vanadate complex was more finely divided and remained longer in suspension. Varying the concentration of the ammonium molybdate had no apparent effect on the separation of impurities or on the formation of the morphine-molybdate-vanadate complex. It is necessary that the final volume does not exceed 3.0 to 3.2 ml., or otherwise less morphine-molybdate-vanadate complex will separate out. After the complex has separated, the solution may be diluted to a volume suitable for nephelometric measurements.

Tests were made to show the relatively high concentration of morphine-molybdate-vanadate complex which never separates out of solution. After allowing the turbid solution to set for several days, it was centrifuged, and the supernatant

fluid removed by decanting. Both the supernatant fluid and the precipitate were tested with sodium carbonate and phenol reagent. When the final volumes in both were made equal, the color in the supernatant fluid was darker than that from the precipitate. No color was obtained from the reagents in the absence of morphine. The evidence is clear that the amount of morphine precipitated as the morphine molybdate vanadate complex depends on a number of factors, such as the acid concentration, the volume in which it is precipitated, the amount of impurities not removed, and the temperature of the solution. These factors must be carefully controlled before the method can be used satisfactorily as a quantitative method.

No turbidity has been obtained in any sample of urine free of morphine or other commonly used drugs. The presence of quinine in urine gives a turbidity which separates out immediately as a yellow flocculent precipitate. Pyrimidon (4 dimethyl amino antipyrine) gives a turbidity similar to morphine. After standing for a number of hours pyrimidon produces a blue green to blue color, which is not obtained by morphine alone. If pyrimidon is suspected, additional tests should be made for its identification. The above test for the detection of morphine in urine should always be confirmed by one or more additional tests. The Marquis or Froehde reagents give characteristic color reactions for morphine when added to a residue obtained by extracting an alkaline urine with ethyl acetate or other organic solvents.

**B Quantitative Procedure** This principle may be used quite satisfactorily in determining semiquantitatively very small amounts of morphine. A series of test tube standards were prepared by adding varying amounts of

TABLE III  
TURBIDITY OBTAINED FROM VERY SMALL AMOUNTS OF MORPHINE

AMOUNT OF MORPHINE ADDED TO 25 ML URINE	APPEARANCE OF FINAL SOLUTION
mg	
0.00	Clear
0.01	Clear
0.02	Very faint trace
0.03	Definitely positive
0.05	Strong
0.07	Stronger
0.09	Much stronger
0.12	Very strong

morphine to 25 ml of morphine free urine and then extracting each sample twice with ethyl acetate, followed by the usual preparation of the morphine molybdate vanadate complex. The amount of morphine present in the urine of most addicts was so high that 10 ml urine was usually sufficient for a satisfactory comparison with a prepared standard. The unknowns were allowed to stand at least two hours before they were compared with a standard. When the turbidity was very high, it was either diluted sufficiently to match with an appropriate standard, or a stronger standard was prepared. If a turbidity is very high, it is not easy to determine small differences in morphine concentrations. Table III shows the relative turbidity obtained by using very small amounts of morphine.

Further attempts were made to make the turbidity method quantitative. The procedure for morphine extraction described by Pierce and Plant was used in the following experiments. The final residues were treated similarly to those obtained from the ethyl acetate extractions. The morphine-molybdate-vanadate complex separated out as fine, white particles which remained in suspension for a long period of time. The presence of excess amounts of impurities caused the morphine compound to coalesce and precipitate faster. Efforts were made to compare the turbidity with an aqueous morphine standard similarly prepared, using a Bausch and Lomb nephelometer and a "Cenco" telophotometer. One hour after the addition of ammonium vanadate the final volume was diluted to 10 ml. and compared with a standard. Satisfactory results were obtained for very small amounts of morphine (Table IV).

TABLE IV  
RECOVERY OF MORPHINE ADDED TO URINE

URINE VOLUME	MORPHINE ADDED TO URINE	MORPHINE RECOVERED	EXPERIMENTAL ERROR
ml.	mg.	mg.	mg.
50	0.00	0.000	0.000
50	0.05	0.050	0.000
50	0.10	0.097	-0.003
50	0.10	0.098	-0.002
100	0.10	0.099	-0.001
150	0.10	0.091	0.000
50	0.10	0.098	-0.002
50	0.10	0.088	-0.012
25	0.10	0.095	-0.005
65	0.10	0.100	0.000

The recovery of morphine added to urine by means of the liquid-liquid extraction process followed by further purification with acid and alkaline extraction. The morphine-molybdate-vanadate complex was prepared as usual. After standing for forty-five minutes, the volume was adjusted to 10 ml., and a comparison made with a similarly treated aqueous morphine standard by means of a nephelometer.

When the amount of morphine exceeded 0.25 mg., unsatisfactory results were obtained. The morphine-molybdate-vanadate particles clumped together into large particles which precipitated rapidly. Under such conditions it was not possible to make satisfactory comparisons with a standard. The turbidity obtained from urine specimens always precipitated more rapidly than that from aqueous morphine solutions.

Attempts were made to stabilize the turbidity by the addition of ghatti gum or arabic gum. Both of these substances prevented the formation of any turbidity.

When ordinary stopcock grease was used as the lubricant on the extractor and separatory funnels, low recoveries were often obtained. When the lubricant was replaced by glycerol, less morphine was lost. Considerable grease was always extracted by the organic solvents and was carried over in the final residue, thus preventing the complete transfer of morphine from the evaporating dish. Small amounts of glycerol do not interfere with the formation of the turbidity.

# SUMMARY

1. Morphine may be determined quantitatively in concentrations ranging from 0.08 to 3.0 mg. per 100 ml. urine by a new colorimetric method. The morphine is extracted from the urine by the Pierce and Plant procedure, using the continuous liquid-liquid extraction process followed by acid and alkaline extractions. The final residue dissolved in water is further purified by means of permutit. Morphine combines with permutit, while most other reducing substances are removed by washing. The morphine is finally determined colorimetrically after the addition of sodium carbonate and Folin-Denis phenol reagent to the permutit.

2. The determination of morphine in urine by the diazo reaction has been modified and its limitations discussed.

3. The presence of morphine in the urine of morphine addicts may rapidly be determined by making two extractions with ethyl acetate and the subsequent production of a morphine-molybdate-vanadate complex. A turbidity appears which is roughly proportional to the morphine concentration and may be compared with a prepared standard. Morphine concentrations as low as 0.03 mg. in 25 ml. of urine can readily be detected.

4. The turbidity produced by the morphine-molybdate-vanadate complex has been developed into a quantitative method for small amounts of morphine extracted from urine by the Pierce and Plant procedure. A nephelometer is used for comparing the unknown turbidity with a standard.

# REFERENCES

1. Pierce, I. H., and Plant, O. H. Studies of Chronic Morphine Poisoning in Dogs. IV. Excretion of Morphine in Tolerant and Non-tolerant Animals, *J. Pharmacol. & Exper. Therap.* 33: 201, 1932.
2. Lautenschlager, L. Die Diazo-Reaktion des Morphiums, *Arch. d. Pharmazie* 257: 13, 1919.
3. Balls, A. K., and Wolff, W. A. The Determination of Morphine, *J. Biol. Chem.* 80: 379, 1928.
4. Deckert, W. Ein neues, leicht ausführbares Schnellverfahren zur quantitativen Bestimmung kleinster Mengen von Morphin in Harn, Blut und anderem biologischen Material, *Arch. f. exper. Path. u. Pharmacol.* 180: 656, 1936.
5. Fry, E. G., Light, A. B., Torrance, E. G., and Wolff, W. A. Opium Addiction, The Excretion of Morphine by Human Addicts, *Arch. Int. Med.* 44: 862, 1929.
6. Wolff, W. A., Riegel, C., and Fry, E. G. The Excretion of Morphine by Normal and Tolerant Dogs, *J. Pharmacol. & Exper. Therap.* 47: 391, 1933.
7. Loofs, F. A. Ueber die Möglichkeit einer klinisch-chemischen Kontrolle des Morphinismus. Bemerkungen zur Theorie der Morphin-Gewohnung, *Ztschr. f. d. ges. Neurol. u. Psychiat.* 79: 433, 1922.
8. Panse, I. A Simple, Reliable Method for the Detection of Morphine in the Urine of Addicts, *Fortschr. d. Therap.* 8: 629, 1932.
9. Teruuchi, Y., and Kai, S. On the Fate of Morphine Which Has Been Injected Into the Animal Body, *J. Pharmacol. & Exper. Therap.* 31: 177, 1927.
10. Ellinger, F., and Seeger, H. The Determination of Morphine in Excretions, Secretions, and Tissues, *Arch. f. exper. Path. u. Pharmacol.* 174: 160, 1933.
11. To, S., and Rin, A. The Determination of Morphine in the Urine of Opium and of Morphine Addicts and the Practical Application of the Reaction, *Jap. J. Med. Sci., IV, Pharmacol.* 7: 11, 1935.
12. Fehden, O., and Huang, C. H. Microdetection of Morphine and Heroin in Urine and Milk by Spot Method, *Pharm. Monatsh.* 18: 73, 1937.
13. Liang, C. K. A New Method for the Detection of Morphine in the Urine of Opium Addicts, *Chinese M. J.* 51: 211, 1937.
14. Folin, O., and Denis, W. On Phosphotungstic Phosphomolybdic Compounds as Color Reagents, *J. Biol. Chem.* 12: 239, 1912.
15. Whitehorn, J. C. "Permutit" as a Reagent for Amines, *J. Biol. Chem.* 56: 751, 1923.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

## TUBERCULIN TEST, Intracutaneous, Paretsky, M. *Am. Rev. Tuberc.* 38: 81, 1938.

A series of 400 "double" tests has demonstrated that the regional variation of specific skin hypersensitiveness to tuberculin is a factor of a practical significance.

A series of 500 "double" tests has yielded results seemingly indicating that the deterioration of tuberculin does not affect its potency to a degree definitely distorting the outcome of the intracutaneous reaction.

A series of 400 "double" tests has revealed that the "tenacity" of tuberculin undoubtedly exerts a distorting effect on the result of the intracutaneous reaction, depicted in our series by a curve which reached its peak when the 1:100,000 solution of old tuberculin was used, and which came down to its lower levels with the increase of the concentration of the solutions of old tuberculin.

A series of 100 "double" tests has apparently failed to demonstrate a noticeable effect of the size of the wheal produced by the injected tuberculin on the size of the reacting skin area.

A series of 100 "double" tests has given results suggesting a probable somewhat retarding effect of alcohol on the intracutaneous tuberculin reaction.

## PNEUMOCOCCUS TYPING, Concentration Method Applicable to the Neufeld Quellung Reaction in Sputums, Taplin, G. V., Meneely, G. R., and Hettig, R. A. *J. A. M. A.* 111: 410, 1938.

1. The entire specimen of sputum is emulsified by drawing it up in a small (2 c.c.) syringe and mixing it thoroughly in a sterile Petri dish until all large particles are broken up.

2. Into a sterile centrifuge tube is placed 0.5 c.c. of the emulsion, and from 6 to 10 c.c. of physiologic solution of sodium chloride is added, depending on the viscosity of the sputum. This mixture is emulsified by drawing the diluted specimen back and forth in a sterile 10 c.c. pipette.

3. The emulsified specimen is put in the centrifuge at 1,000 r.p.m. for two or three minutes to remove cells and detritus, the bacteria remaining in the supernatant fluid.

4. The supernatant fluid is transferred to another tube, which is put in the centrifuge at 3,000 r.p.m. for ten minutes, after which the clear fluid is poured off, leaving about 0.25 c.c. above the sediment.

5. The bacterial sediment is resuspended in the small remnant of supernatant fluid by shaking, and the resulting suspension is used for the typing tests. Before the suspension is mixed with specific typing sera, two drops of methylene blue are added and mixed with this suspension, the organisms being allowed to stain before the Quellung reaction takes place. This has been used because in many cases when the sera and stains are added simultaneously, the organisms swell before they stain and may be difficult to recognize.

As a result of this procedure, the Quellung reaction takes place quickly, in some cases, immediately. Detection of capsular swelling is rendered much easier by the lack of cells and detritus and by the larger number of organisms in the field (from 10 to 300 per oil immersion field). No injury to the capsule has been observed as a result of the dilution with physiologic solution of sodium chloride and centrifugation. No false positive reactions have been observed. The suspension and washing of sputum in saline solution probably removes the soluble substance that causes agglutination, and failure of the Quellung reaction in other highly concentrated specimens. With this technique highly concentrated suspensions may agglutinate but they never fail to swell.

**GONOCOCCI, A New Contrast Stain for Meningococci and, Sandiford, B. R. Brit. M. J. May 28, p. 1155, 1938.**

Any Gram technique to which the worker is partial may be followed up to the end point of decolorization, but the following is strongly recommended, especially the use of acetone, which is essential in order to obtain complete decolorization of the cell nuclei before counter-staining.

#### Initial Stain

Crystal violet	1 gm.
Alcohol (98 per cent)	20 c c.
Ammonium oxalate (1 per cent aqueous)	30 c c

1. Leave this mixture on the heat fixed film for thirty seconds
2. Flood off with triple strength Lugol's iodine (as used in Jensen's modified Gram stain) and leave on for thirty seconds.
3. Pour off the excess iodine and blot once
- 4 Decolorize with acetone for three or four seconds
5. Wash.
6. Put on the counterstain for two minutes.
7. Flood off with water—do not wash—and blot

#### Contrast Counterstain

Malachite green	0.05 gm
Pyronine	0.15 gm
Distilled water	100.0 c c

Note: There are two varieties of pyronine "B" and "G," the former being almost insoluble in water. Pyronine (Grubler) is apparently pyronine "G," as it is freely soluble in water.

*Keeping Qualities.* The following statements are based on haphazard observations only, during the use of solutions kept in ordinary bottles at room temperatures under 20° C: Crystal violet stain keeps for about a month and deteriorates after six weeks. Counterstain keeps for at least three weeks, possibly longer, 100 cc in a staining pot may be used for at least 150 small smears

**TUMORS, Multiple Primary Malignant, Eisenstaedt, J. S. J. A. M. A. 110: 2056, 1938.**

A man who died in 1936 had been operated upon twenty one years before for adenocarcinoma of the kidney. He later was affected with two other primary cancers in distinct and separate organs, one of which, that of the prostate, produced metastases to the lungs and lymph nodes. All three cancers were diagnosed clinically.

It is the author's belief, after a rather complete survey of the literature, that:

1. Multiple primary cancers are more frequent than are reported.
2. They occur more frequently than chance alone would explain.
- 3 The percentage of multiple primary malignant tumors to be reported in the future will be higher than that recorded in the past, because

(a) More people are reaching advanced years, a period in which the incidence of cancer is generally high. (b) Since the inauguration of cancer clinics and commissions throughout the world, cancer patients have been more concentrated and more thoroughly studied. (c) Better results are being obtained in the treatment of cancer than heretofore. (d) As a result of longer survival after treatment for a single primary tumor, time is afforded for the development of subsequent primary cancers. (e) Autopsies are more widely done and with greater thoroughness than in the past.

4. Some factor, as yet unknown, possibly hereditary or hormonal in nature, plays an important part in susceptibility to malignant disease, and the varied responses to environment in different individuals depend on this unknown element.



**TISSUE:** Staining of Acid-Fast Bacilli in Paraffin Sections, Fite, G. L. *Am. J. Path.* 14: 385, 1938.

The optimal staining of acid-fast bacilli in tissues involves fixation in an alcoholic medium, removal of mercuric deposits with iodine followed by alcohol and sodium thiosulfate, and staining in a 1 per cent solution of new fuchsin in 5 per cent phenol and 10 per cent methyl alcohol.

If a potassium dichromate fixative is used, the period of fixation must be brief and the tissue thoroughly washed, and the section must be treated with potassium permanganate and oxalic acid.

Time and materials are wasted in trying to stain acid-fast bacilli in tissues subjected to prolonged passage in formaldehyde or decalcifying fluids.

To ensure the maximal staining possible under the conditions of fixation, the sections should be stained:

After alcohol fixation, at 20° C.—two to eight hours,  
 at 37° C.—one to four hours,  
 at 60° C.—thirty minutes to two hours, or  
 at 90° C. (steaming)—five minutes;

After all other fixation, at 20° C.—sixteen to twenty-four hours,  
 at 37° C.—twelve to sixteen hours,  
 at 60° C.—eight to twelve hours, or  
 at 90° C.—five minutes.

Under good conditions bacilli may be adequately stained in much shorter periods of time than these. Bacilli not stained by fuchsin in the maximum times given will not be stained by further treatment. This is the point to which the duration of staining is best carried. Prolonged staining at room temperature is preferred.

New fuchsin as a dye for acid-fast bacilli is much superior to basic fuchsin.

When, in Zenker fixed tissues, fuchsin fails to stain bacilli resistantly, they may sometimes still be stained by Gram's stain, by the acid-fast method using crystal violet, or by the fuchsin-formaldehyde method here recorded.

The following stain is recommended:

New fuchsin	1 gm.
Phenol crystals	5 gm.
Methyl alcohol	10 c.c.
Distilled water to make	100 c.c.

The dye is wholly dissolved in the mixture of alcohol and phenol, and the distilled water is added, not too rapidly. The solution should be clear and not require filtration. Fuchsin is so readily precipitated by a wide variety of substances that to maintain the full strength distilled water of high purity must be used. The solution preserves indefinitely, but it should be remembered that evaporation of 5 per cent by volume, which will largely be alcohol, will precipitate dye to a much greater proportion. Solutions left in Coplin jars lose strength rapidly.

**TUBERCULOSIS, Bromsulphalein Test and Blood Cholesterol in Pulmonary Tuberculosis,** Levinson, S. A., and Siegal, H. A. *Am. Rev. Tuberc.* 38: 229, 1938.

The bromsulphalein liver function test and blood cholesterol were studied in 28 cases of far-advanced pulmonary tuberculosis. These tests can be used as an index in the prognosis of the disease condition.

The combination of hypocholesterolemia with bromsulphalein retention indicates extreme gravity.

The hypocholesterolemia and bromsulphalein retention may be related to the functional activity of the reticulo-endothelial system, as evidenced by the greater demand for cholesterol by the organ. The degree of stimulation of the reticulo-endothelial system can be measured by the amount of bromsulphalein dye retention. The greater the stimulation the greater the retention of the dye.

**TUBERCULIN:** Mantoux Tests With the Gottschall-Bunney Diluent for, Clark, E. Am. Rev. Tuberc. 38: 270, 1938.

A total of 1,648 Mantoux tests were performed from December 15, 1936, to March 19, 1937, comparing tubercula freshly diluted in saline with that ready diluted in the diluent of Gottschall and Bunney.

The results of these tests seem to indicate that the ready diluted tuberculin is as efficient as the freshly diluted tubercula and retains its potency when kept at room temperature for a period of at least one month.

Tubercula ready diluted 1:10,000 and incubated thirty days at 37° C. showed some deterioration evidenced by a difference in induration of the Mantoux reaction. Although the sizes of the reactions were comparable, the induration was not quite as hard as that caused by freshly diluted material.

**RETICULOCYTES, Demonstration of, With Wright's Stain, Kitchen, S. F. Stain Technol. 13: 107, 1938.**

A 0.3 per cent solution of the stain in absolute, acetone free methyl alcohol was used, and in making most preparations a thin, even film of the dye was spread on a glass slide. A small drop of blood was placed on a cover slip which was then inverted on the slide and the preparation sealed with vaseline.

The reticulocytes showed up quite as numerous as they did when brilliant cresyl blue was similarly applied, although a little more slowly. In such preparations the reticulum appeared not infrequently in the form of filaments which usually had a tendency to interlace, sometimes quite densely, so that there was a compact mass in the center of the red cell, or perhaps eccentrically placed. At other times the interlacing network was more loosely formed and traversed the cell like a band. Most frequently the reticulum was observed to assume a different morphologic character. This was a branching form which gave it an appearance not unlike moss. In such instances it was fuzzy and much less well defined. A reticulocyte might exhibit as little as a single small Y shaped branch, or the branching forms might be scattered quite profusely throughout the cell. Regardless of what form the filamentous reticulum assumed, one or more metachromatic globules, varying considerably in size and exhibiting Brownian movement, were occasionally observed. In some instances these globules were isolated and at other times in intimate relationship with the filamentous material.

Dry slide preparations were made by spreading a moderately heavy film of the 0.3 per cent dye solution on a glass slide, and when this was dry, superimposing a blood smear thin enough so that the individual cells were separated. Wright's stain was then used as a counter stain in the usual way. In these preparations the reticulum usually appeared as granules of varying size and number. The intensity of the staining reaction varied greatly, as well as the amount of reticulum so precipitated. In some cells there was observed a light sprinkling of very fine granules, while others were packed with heavily stained, short, thick rods. Still other reticulocytes might contain a relatively small number of larger, coarse granules which were frequently irregular in outline.

**HEPATOSPLENOGRAPHY: Histopathologic Study of Tissues of 65 Patients Injected With Thorium Dioxide Sol for, Yater, W. M., and Whitmore, E. R. Am J M Sc 195: 198, 1938**

Necropsies have been performed on 64 patients at intervals of a few days to three years after the injection of an average dosage of 75 cc of thorotrast for the purpose of making hepatosplenograms. In no case was there any evidence of injury to the tissues nor cellular reaction that could be ascribed to the presence of the thorium dioxide.

A subcutaneous nodule was excised from the arm four years and five months after the injection of thorotrast accidentally into the subcutaneous tissues. Thorium dioxide was walled off by dense hyaline connective tissue, the nodule resembling those found in the lungs in nodular silicosis. Nowhere was there any evidence of injury to the tissues nor cellular reaction other than the primary reaction resulting in the walling off of the thorium dioxide.

## REVIEWS

Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

### Digrafía\*

THIS monograph describes a new method of roentgenography for the study of moving organs. The principle involved is briefly a series of narrow parallel slats which completely cover a film, and which are hinged at the ends in such a way that every other slat may be opened at a given time for one exposure, and at any desired interval thereafter the other half of the slats are opened to make the second exposure. One half may, therefore, be open during systole of the heart and the other half during diastole—both of these being, therefore, recorded on the same film, permitting accurate comparison of area, range of motion, etc. The principle can be extended in such a way as to include even more exposures on the same film. The method would appear to have distinct possibilities and certain definite advantages over roentgen-kymographs.

### Sulfanilamide Therapy of Bacterial Infections†

PERHAPS, not since the discovery of salvarsan has the introduction of a new chemotherapeutic agent aroused more interest than that following the introduction of sulfanilamide.

Despite the fact that its mode of action and its limitations are still far from clearly understood, that this compound possesses remarkable potentialities is becoming more and more evident so that this volume, representing a careful survey by workers of experience, is one to be read with the greatest of interest and profit.

The book begins with a comprehensive survey of the literature dealing with the sulfonamide compounds, including a survey of their experimental and clinical use. This is followed by a section dealing with the authors' *in vitro* experimental studies of sulfonamide and their results in the treatment of streptococcal and pneumococcal infections in animals and their experience in the treatment of pneumonia and streptococcal meningitis in man.

Section III discusses the mechanism of the action of sulfonamide. Section IV discusses general therapeutic considerations and the criteria of therapeutic efficiency.

An addendum of 18 pages reviews studies which appeared after the manuscript went to press.

While presenting a comprehensive survey of the literature it must not be thought that this book is a mere compilation of abstracts. On the contrary, the authors on every page show evidence of ability to evaluate the studies of others as well as their own and of a clear concept of the complexities involved in the study and evaluation of chemotherapy.

This is not only a timely book, a thoughtful book and one presenting a well-balanced consideration of experimental and clinical evidence; it is a valuable contribution of marked importance.

This book should be read by all who are interested in the chemotherapeutics of disease.

\*Digrafía. By Guido Pollitzer, M.D. 104 pages, 41 illustrations. Imprenta Amorrotu, Buenos Aires.

†Sulfanilamide Therapy of Bacterial Infections, With Special Reference to Diseases Caused by Hemolytic Streptococci, Pneumococci, Meningococci and Gonococci. By Ralph R. Mellon, M.D., Director, Institute of Pathology, Western Pennsylvania Hospital; Paul Gross, M.D., Pathologist to the Institute; and Frank B. Cooper, M.S., Research Chemist to the Institute. Cloth, 398 pages, \$4.00. Charles C. Thomas, Springfield, Ill.

### The Foot

THAT this book should have reached a second edition is not surprising, for it presents in an eminently simple and practical manner a discussion of ailments, the importance of which has not always been emphasized to the practitioner.

The present edition is enriched by a considerable increase in the number of illustrations, and also by a revision and addition to the text.

As before, this book may be recommended as one of practical value to the practitioner.

### Handbook of Practical Bacteriology†

THIS deservedly popular English text remains, as before an eminently practical guide to bacteriologic laboratory work and as such can be well recommended.

It has been thoroughly revised to embody the changes and advances which have taken place in the four years since the last edition.

### Clinical Chemistry and Practical Medicine‡

IT IS not surprising to find that this book has reached a second edition, for it is an excellent and practical exposition of particular value to the practicing physician.

Biochemistry has become an important and almost indispensable adjunct to clinical medicine. In this book the physician will find a clear cut and comprehensive discussion of its practical application to the problems of everyday medicine.

### Clinical Atlas of Blood Diseases§

THOUGH small in size, this volume contains a wealth of readily assimilable information and that it has reached a fourth edition is not surprising.

Five new plates have been added in the present edition, and the text has been revised in accordance with the advances in the field of hematology.

The physician, the pathologist, and the laboratory worker will find this book a valuable addition to the working reference library.

It can be recommended with enthusiasm.

### The Troubled Mind||

AS STATED in the preface, the purpose of this book is to present in simple terms a description of mental and nervous diseases, with particular emphasis on the psychopath as a disturber of the public peace.

\*The Foot. By Norman C. Lake, M.D., M.Sc., F.R.C.S., Senior Surgeon and Lecturer on Surgery, Charing Cross Hospital, etc. Cloth, ed. 2, 366 pages, 113 illustrations. \$4.50. William Wood & Co., Baltimore, Md.

†Handbook of Practical Bacteriology. By T. J. Mackie, M.D., D.P.H., Professor of Bacteriology, University of Edinburgh, and J. E. McCartney, M.D., D.Sc., Director of Research and Pathological Services, London County Council. Cloth, 586 pages. \$4.00. William Wood & Co., Baltimore, Md.

‡Clinical Chemistry and Practical Medicine. By C. P. Stewart, Lecturer in Biochemistry, University of Edinburgh, and J. E. McCartney, M.D., D.Sc., Director of Research and Pathological Services, London County Council. Cloth, 372 pages, 38 illustrations. \$4.00. William Wood & Co., Baltimore, Md.

§Clinical Atlas of Blood Diseases. By A. Piney, M.D., M.R.C.P., Consulting Physician, International Clinic, Tunbridge Wells, etc., and Stanley Wyard, M.D., M.R.C.P., Physician, the Royal Cancer Hospital, London, etc. Cloth, ed. 4, 127 pages, 42 illustrations, 38 in color. \$4.50. P. Blakiston's Son & Co., Philadelphia, Pa.

||The Troubled Mind. A Study of Nervous and Mental Diseases. By C. S. Bittern, M.A., M.D., F.A.C.P., M.R.C.S. (Eng.). Cloth, 520 pages. \$3.50. Williams & Wilkins Co., Baltimore, Md.

The book is divided into eight main sections: I. Fixed Ideas and Reactions; II. Psychoneurosis—Its Manifestations; III. Psychoneurosis—Its Nature and Causes; IV. Traumatic Hysteria; V. Clinical Types of Inhibition; VI. Sundry Disorders; VII. Mental Illness; and VIII. The Patient's Role in Therapy.

It is quite evident from the text that the author has had an extensive and comprehensive experience, and also that he has the ability to discuss a complex subject in a clear and understandable fashion.

A glossary is appended for the lay reader.

Those who read this book will undoubtedly have a better understanding of the mental mechanism and a clearer appreciation of the disturbances to which it is subject.

### A Textbook of Bacteriology\*

THIS second edition, written in considerably fewer pages than the average textbook on bacteriology, seeks to present only the fundamental and definitely accepted phases of the subject. The book is concise, practical, and sufficiently complete for the beginner. This is quite in keeping with the feeling of the author: that the beginning student or practitioner cannot assimilate the subject matter given in the more inclusive textbooks.

### Pathological Technique†

EVERY laboratory must appreciate the appearance of Dr. Mallory's new presentation of pathologic techniques.

"I have undertaken the writing of this book as a result of many requests for a modern presentation of accepted pathological techniques," Dr. Mallory writes in beginning the preface to the book. "Technical methods are constantly changing in pathology as in other branches of science. The wisest plan in issuing a book like this seems to be to retain the best of the past and to add only the most promising methods of the present." The author has done just that, and in the same degree of excellence that characterized the old Mallory and Wright of more than a decade ago. The new publication retains only the accepted techniques of the past, and includes the more modern procedures which up until now have been accessible only in the foreign literature or individual publications.

For years the pathologic laboratory has felt the need for such a book as Dr. Mallory's, and a cordial welcome is assured.

### General Bacteriology‡

A COMPREHENSIVE textbook of general bacteriology intended especially for the medical student. The fact that the book has reached a twelfth edition speaks for its usefulness. Dr. Burrow's revision considers the field of bacteriology from the broader point of view, especially clinically, leaving, as indicated in the preface, the more special laboratory procedures to manuals of bacteriology.

\*A Textbook of Bacteriology. By Thurman B. Rice, A.M., M.D., Professor of Bacteriology and Public Health, Indiana University School of Medicine. Cloth, 554 pages, \$5.00. W. B. Saunders Co., Philadelphia, Pa.

†Pathological Technique. By Frank Burr Mallory, A.M., M.D., S.D., Consulting Pathologist to the Boston City Hospital. Cloth, 397 pages, \$4.50. W. B. Saunders Co., Philadelphia, Pa.

‡General Bacteriology. A Textbook. By Edwin O. Jordan, Ph.D., Formerly Professor of Bacteriology, University of Chicago. Revised by William Burrows, Ph.D., Assistant Professor of Bacteriology, University of Chicago. Cloth, ed. 12, 825 pages, \$6.00. W. B. Saunders Co., Philadelphia, Pa.

# The Journal of Laboratory and Clinical Medicine

Vol. 24

JANUARY, 1939

No. 4

---

## CLINICAL AND EXPERIMENTAL

---

### JUVENILE DIABETES MELLITUS

---

#### A COMPARATIVE STUDY OF STANDARD INSULIN, CRYSTALLINE INSULIN, PROTAMINE INSULIN, AND HEXAMIN INSULIN

---

HENRY M. FLINBLATT, M.D., BROOKLYN, N. Y.

---

#### CRYSTALLINE INSULIN

IN 1926, Abel<sup>1</sup> prepared a crystalline insulin. This preparation of insulin, because of expense of production and technical difficulties, was unsuitable for use in our group of cases.

In 1934-35, Sahyun<sup>2</sup> produced a crystalline preparation of insulin with a low nitrogen content and an assay value of 25 to 25.5 units per mg. dry weight.

A group of 11 cases was selected from the diabetic clinic and admitted to the hospital for the study with crystalline insulin.<sup>3</sup> Six of the patients were between 10 and 17 years of age, 4 were between 20 and 25, and 1 was 50 years old.

The diets and insulin dosage were constant and identical with the patients' regular routine. The routine diets varied with relation to size, weight, and activity of the individual patient.

This study revealed that low blood sugars are sustained four hours longer with crystalline insulin than with standard insulin.

#### PROTAMINE INSULIN<sup>4</sup>

Out of a group of 400 active, ambulant, diabetic individuals treated with protamine insulin for over eighteen months, the first small group studied was selected for this report.

Diets and insulin dosage had been established. Blood sugar was determined at two-hour intervals, and comparative graphs of the blood sugar levels on regular and protamine insulin were made throughout the experimental period.

The diets were prescribed to conform with the habits of the patient and the family and included a late meal at bedtime. The low ratio was 3 carbohydrates to 1 of protein and 1 of fat; the high ratio 5 carbohydrates to 1 of protein and 1 of fat. The entire group showed a gain in weight, and in some the diet had to be restricted to prevent obesity.

The unit value of protamine insulin was found to be greater and more lasting than the unit value of standard insulin. Our dosage was calculated upon the basis of 80 per cent of the dose of standard insulin.

We were pleased by the absence of ketonuria and the decreased incidence of lipemia in this group under protamine insulin therapy, especially as the presence of acetone breath and acetoneuria was almost a constant finding in these diabetics controlled with standard insulin.

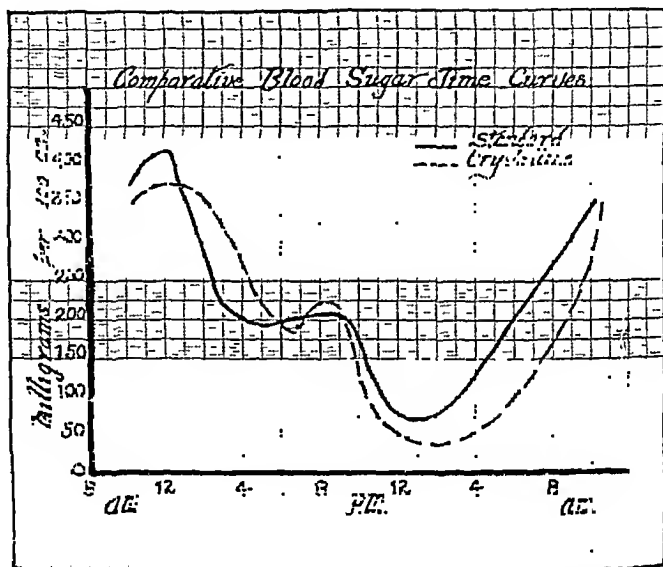


Fig. 1.—Composite graph of 11 cases showing blood sugar levels at two-hour intervals under administration of standard insulin and crystalline insulin.

Obviously, this insulin precipitate is remarkably effective in stimulating the completion of fat metabolism and thus eliminating ketonuria from diabetes.

Our juvenile diabetics frequently showed blood sugar levels below 60 mg., and were entirely free of symptoms at such levels. This level was, therefore, taken as a routine safe and comfortable margin for those under protamine insulin therapy.

A modification of this level was considered for those patients of middle age or over, with or without cardiac disease or hypertension, because this type commonly reacted at sugar levels in the neighborhood of 70 mg. For these cases a low level of 100 mg. has proved most satisfactory.

Blood sugars were examined at two-hour intervals throughout the twenty-four hours, and an average was determined for each case and for the group.

The curve of periodic variations in a group of 30 cases of juvenile diabetes revealed a continuous sustained drop which reached its lowest level at midnight.

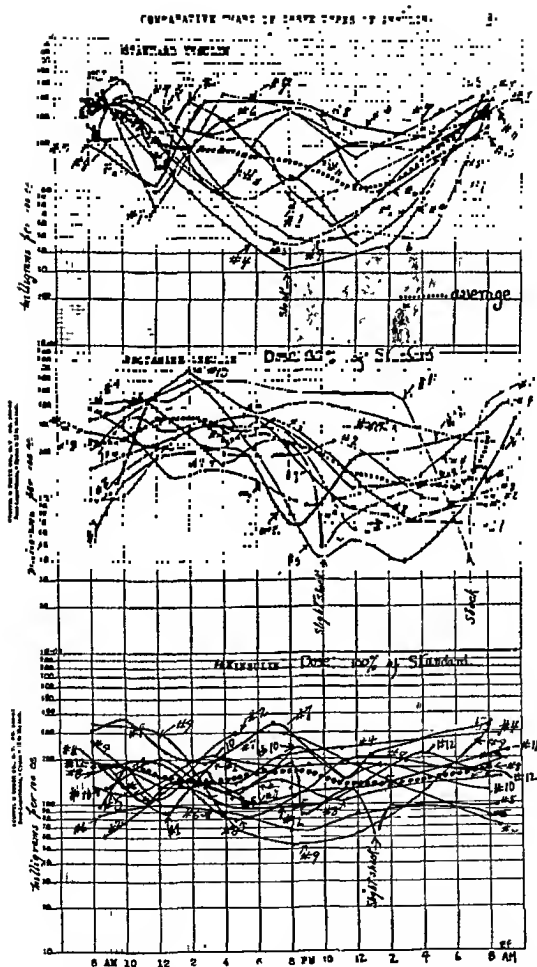


Fig. 2—Eleven cases treated successively with standard protamine and hexamine insulin. Same number refers to same patient in each graph.



The average blood sugar level was 177 mg. Claude Bernard's threshold value of 170 mg. was decided upon as the average optimum diabetic threshold level.

During the first twenty-four-hour period after injection of protamine insulin, a drop in blood sugar began during the fifth hour and continued to the end of the twenty-four hours. During the second twenty-four-hour period, the sugar curve showed a continuous rise after the morning injection which reached a plateau in five hours and was followed by a drop which reached its lowest level twenty-four hours after injection.

The average curve on protamine insulin therapy, as shown on the graph by the dotted line, demonstrates a sinelike form. This makes it possible for the application of a formula which would show a direct relationship between the high and low peaks and the daily average levels.

Protamine insulin adjustment was established at the end of the third day. The unit value of protamine insulin was found to be 20 per cent greater than the unit value of regular insulin. In the group studied, the intereipient use of standard insulin conflicted with the efficiency of protamine insulin and was eliminated.

The introduction of the protamine preparation of insulin was a tremendous contribution to the treatment of diabetes. The practicality of a single daily injection encouraged both the patient and the physician to favor insulin therapy.

There has been, however, some hesitancy on the part of the medical profession to fully utilize this new therapeutic factor because of the following reasons:

1. Fear of allergic manifestations. Such reactions have occurred in our group and are decidedly more common with protamine insulin than with standard insulin.

2. Delayed response. On the basis of the average, there is an immediate response in a lowering of the blood sugar with standard insulin. With protamine insulin, the response is not evident until the sixth hour. Because of this delayed response, the rise in blood sugar caused by breakfast is not checked by protamine insulin. Most investigators found it necessary to use intereipient doses of standard insulin to check this early rise. This often resulted in a summation effect expressed in insulin shock.

3. Insulin shock. Attempts to lower the average blood sugar levels by increasing the dose of protamine insulin (because of the sinelike character of the blood sugar curve) also lowers the low points and results in hypoglycemia.

4. Character of the precipitate. The precipitate, on standing, packs, and it is only with difficulty that it can be shaken into a homogeneous mixture. Strengths higher than U-80 clog the 27 gauge needle, and the larger needles are objectionable.

5. Local reactions. Annoying local reactions occur at site of injection of more than 1 c.e. protamine insulin. They are in the form of hard, firm, inelastic nodules, which persist two or three days and are gradually absorbed without discoloration.

6. Character of shock. Shocks caused by protamine insulin, as compared with those produced by standard insulin, last longer and do not respond as quickly to the ingestion of carbohydrates.

## HEXAMINE INSULIN\*

In the course of our investigations, one of my co workers, Dr Warbuton, developed a compound of insulin and hexamethylene tetramine using 0.25 grams of hexamine to 1000 units of insulin.

The particles of the precipitate were very fine and could readily pass through the 27 gauge needle in strengths as high as U 1000.

We then found that this precipitate could be dissolved on either the acid or alkaline side and that it would reprecipitate at the pH of the body tissues.

This preparation could be buffered and was nonirritating. The alkaline buffered solution was as stable as protamine insulin and, when cleared, could be used in any concentration.

Fourteen of the most severe cases of juvenile diabetes were selected for the study of hexamine insulin. This group included those patients treated with standard, crystalline, and protamine insulin.

The dosage of insulin and diet which we have found necessary to maintain these patients in a satisfactory mental and physical state was used as a criterion for this study. In each case the hexamine insulin was administered in a single daily dose. Blood sugar determinations were made at two and four hour intervals throughout the twenty four hours.

An average of 12 cases on regular insulin showed a drop of 200 mg in blood sugar four hours after the first injection and another drop of 200 mg six hours after the second injection.

Protamine insulin resulted in a low morning peak of 120 mg at 6:00 A.M., and blood sugar levels of 230 mg and 250 mg at 8:00 A.M. The first eight hours after injection of protamine insulin were characterized by high sustained levels, the average being 350 mg.

With hexamine insulin the average morning blood sugar level was 180 mg. In general, with this insulin the average curve fluctuates between 140 and 170 mg throughout the entire day.

The absence of ketonuria was as striking with hexamine insulin as with protamine insulin. This insulin also has a specific effect in decreasing polyuria in those patients who complained of it both with standard and with protamine insulin. Shocks caused by hexamine insulin respond immediately to the ingestion of small amounts of carbohydrates.

A single dose of hexamine insulin may be substituted for two to four divided doses of standard insulin. One dose of hexamine insulin produces a blood sugar curve similar to the one produced by four divided doses of standard insulin, but with decided constriction in the range fluctuations of blood sugar.

All the hexamine insulin used was prepared by Edgar A. Ferguson.  
The author is indebted to Dr. Alpert for his clinical and laboratory assistance.

## REFERENCES

1. Abel: Crystalline Insulin, *J. Pharmacol. & Exper. Therap.* 31: 63, 1927.
2. Sahyun, M.: Modification of Folin and Wu's Method for Sugar Determination, *J. Lab. & Clin. Med.* 21: 1089, 1936.
3. Feinblatt, H. M., and Ferguson, E.: Report of Comparative Blood Sugars in Diabetes With Standard Insulin and With New Improved Insulin, *M. Rec.* 145: 67, 1937.
4. Feinblatt, H. M., Alpert, B., and Ferguson, E.: Protamine Insulinate in Juvenile Diabetes, *M. Rec.* 145: 366, 1937.
5. Feinblatt, H., Warbuton, R., Alpert, B., and Ferguson, E.: Hexamine Insulin. Paper now in preparation.

# ATMOSPHERIC POLLEN OF NASHVILLE, TENN.\*

EVANGELINE BOWIE, M.D., NASHVILLE, TENN.

NASHVILLE is located in the middle Tennessee Basin. A record of the pollen content of the air was obtained from an elevated station on the campus of Peabody College, which is located in the western residential section of the city. This elevation is in the path of winds bringing in pollen from the wooded hills around the city.

The technique employed for catching atmospheric pollen, and the method of counting are described by Wodehouse.<sup>1, 2</sup> Melted fuchsin glycerin jelly was placed on slides to cover an area equal to a No. 0 cover glass. Exposures were made in a horizontal position on the roof of a five-story dormitory. Slides removed were warmed, and in order to prevent flattening of the pollen grains, it was often necessary to add an additional drop of melted jelly before applying the cover glass.

The relative abundance of fall elm pollen is shown in the accompanying graph for the fall of 1936 and 1937. The source of this pollen is a fall flowering elm, *Ulmus serotina* Sargent, which is native to middle Tennessee and Kentucky. Gattinger<sup>3</sup> states that it was discovered in 1878 near the grounds of Vanderbilt University. The increase in amount of fall elm pollen and the decrease of ragweed pollen during 1937 as compared with that of 1936 may be explained by a difference in rainfall. During the growing season of 1937, the rainfall was sufficient to keep the trees in heavy foliage, and the effect of shade may have been a factor in the decrease of ragweed.

Black and Durham<sup>4</sup> state that fall blooming elms of the South may cause some perplexing problems during the ragweed season. Their chart of pollen counts in Dallas, Texas, shows fall elm pollen from cedar elm, *Ulmus crassifolia* Nutt., reaching a peak during the second week of September, 1929. Chinese elm, *Ulmus parvifolia* Jacq., which flowers in the summer or fall, has been introduced into the United States, and may become of some importance in the future as a source of elm pollen.

The first grains of giant ragweed (*Ambrosia trifida*) are caught around August 15. Later the grains of dwarf ragweed (*A. elatior*) appear.

*Artemisia* pollen is one of the most abundant varieties of fall pollens. *Artemisia annua* is found in the city and is commonly called Sweet Annie, sweet wormwood, or annual wormwood.

Chenopod includes the pollen of lamb's quarters (*Chenopodium album*), Mexican tea (*C. ambrosioides*), Jerusalem oak (*C. botrys*); pigweeds (*Amaranthus hybridus* and *A. spinosus*); and burning bush (*Kochia scoparia* Schrad.).

\*Read before the Botany Section at the Forty-First Meeting of the Tennessee Academy of Science, Nashville, Tenn., Nov. 26, 1937.

Received for publication, April 4, 1938.

Grass pollen is found in small amounts from spring to frost. Blue grass (*Poa pratensis*) and orchard grass (*Dactylis glomerata*) flower in April and May. Rye grasses (*Lolium perenne* and *L. multiflorum*) are planted for a winter cover, and bloom in May and June. Bermuda and Johnson grass bloom during the summer and fall. Other species of grass which are contributory to hay fever occur in this region.

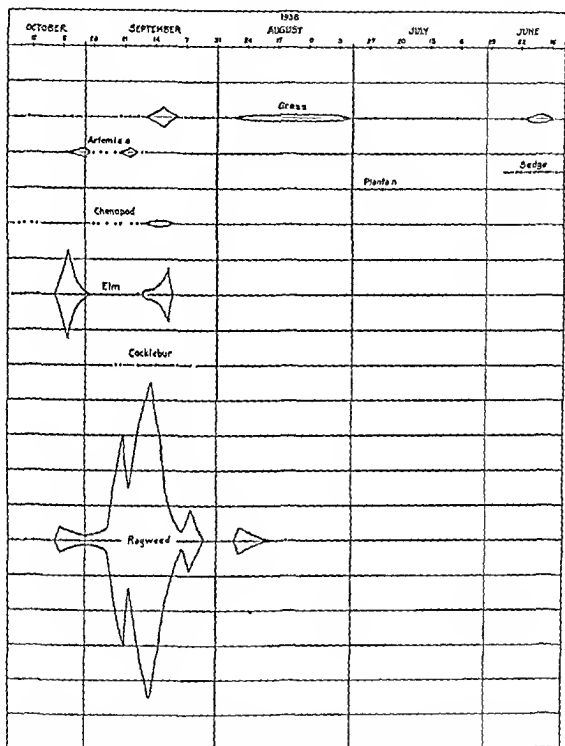


Chart 1—Record of the atmospheric pollen of Nashville, Tenn., during the summer and fall of 1936 and the year of 1937. The distance between the horizontal lines represents 16 grains of pollen caught on an area of 35 sq cm during twenty-four hours (Continued on pages 344 and 345).

During 1937 the percentage of tree pollens was larger than that of grass and weed pollens. Spring rains and rapid changes in temperature account for sharp peaks and depressions of tree pollens.

Elm pollen is one of the earliest spring pollens. Some authors may be found shedding pollen during warm days in January. Winged elm (*Ulmus alata* Michx.), American elm (*U. americana*), and slippery elm (*U. fulva*



Birch trees occur very sparingly and the pollen is negligible.

A large quantity of beech pollen is blown into the city from the surrounding woods.

Hackberry trees are very abundant in Nashville. Pollen of the two species, *Celtis occidentalis* and *C. mississippiensis* Bosc., was not differentiated.

Maclura: Osage orange (*M. pomifera* Schmeid.) sheds a large amount of pollen during May.

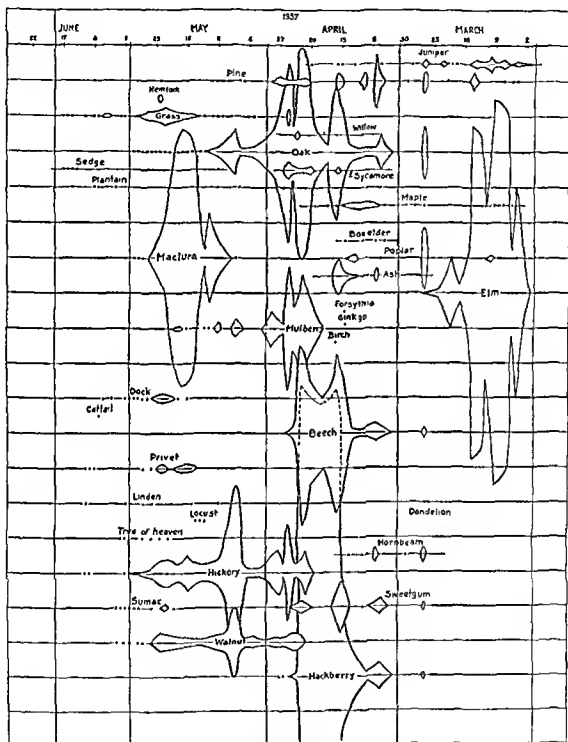


Chart 1.—Cont'd

Mulberry: Under this head are included white mulberry (*Morus alba*), red mulberry (*M. rubra*), and paper mulberry (*Broussonetia papyrifera*). The pollen of paper mulberry predominates. Bernton<sup>6</sup> has shown that osage orange, white and red mulberry, and paper mulberry have a generic relationship in causing hay fever.

Walnut and hickory pollens closely parallel each other in time of appearance. Black walnut (*Juglans nigra*), white walnut (*J. cinerea*); shagbark

hickory (*Carya ovata* Mill.), mockernut (*C. alba*), and pignut (*C. glabra* Mill.) are found in the city and surrounding woods.

Sycamore: *Platanus occidentalis* and *P. orientalis* are along the driveways of the campus.

Privet (*Ligustrum vulgare*) is used on the campus for hedges, and allowed to bloom profusely.

#### SUMMARY

A study of the atmospheric pollen of Nashville, Tenn., during the summer and fall of 1936 and the year of 1937, is presented in the form of a chart and briefly discussed.

Attention is called to the presence of fall elm pollen in order that its importance in allergic conditions may be considered.

#### REFERENCES

1. Wodehouse, R. P.: Pollen Grains, New York, 1935, McGraw-Hill Book Co.
2. Wodehouse, R. P.: Atmospheric Pollen, *J. Allergy* 4: 220, 1933.
3. Gattinger, A.: The Flora of Tennessee and Philosophy of Botany, Nashville, 1901, Press of Gospel Advocate Publishing Co.
4. Black J. H., and Durham, O. C.: Elm Pollen as a Complicating Factor in Hay Fever, *J. Allergy* 1: 501, 1930.
5. Acquarone, P., and Gay, L. N.: A Survey of the Pollen Flora in Baltimore During 1929, *J. Allergy* 2: 336, 1931.
6. Bernton, H. S.: Hay Fever and Asthma Caused by the Pollen of the Paper Mulberry, *J. LAB. & CLIN. MED.* 13: 829, 1928.

### THE EFFECT OF COPPER AND IRON UPON THE SECONDARY ANEMIA OF THERAPEUTIC MALARIA IN GENERAL PARESIS\*

PURCELL G. SCHUBE, M.D., BOSTON, MASS., AND  
BLAKE D. PRESCOTT, M.D., HARTFORD, CONN.

THE value of copper as a supplement to iron in the regeneration of hemoglobin was demonstrated first on anemic rats in 1928 by Hart, Steenbock, Waddell, and Elvehjem.<sup>1</sup> They showed rather conclusively that soluble inorganic iron salts in the presence of copper could be used to stimulate hemoglobin formation. Since the publication of this work, a number of other individuals have studied the effect of copper and other metals in conjunction with iron on hemoglobin production in anemic animals and in man. Most of these studies have verified the conclusions of Hart so that today, although it has been demonstrated that copper is not the only element which, when given with iron, stimulates hematopoiesis, practically everyone is in agreement that copper can be a most active catalyst in the stimulation of hemoglobin synthesis.

In the course of our work on the treatment of general paresis with malaria, we have been frequently annoyed and worried by the secondary anemias which almost invariably resulted. At times these anemias were exceedingly alarming, not only in their intensity, but also in the persistency with which

\*From the Boston State Hospital, and the Neuro-Psychiatric Institute and Hospital of the Hartford Retreat.

Received for publication, April 14, 1938.

they remained after the termination of the malaria, and irrespective of the institution of ordinary iron or liver therapy. The combination of copper with iron was instituted in some of these cases, and the results being sufficiently promising, it was decided to study the problem carefully. It is the purpose of this paper to present the results of this study.

#### METHOD

In each instance the procedure was the same. The hemoglobin and red cell counts were determined weekly before, and throughout the administration of the copper and iron. The drugs\* were always administered orally in gelatinous capsules, a capsule being given three times daily. Each capsule contained 32 mg of iron and 10 mg of copper. The red blood cells were counted by the usual pipette and counting chamber method. The hemoglobin was determined chemically.

The controls were of three types. (1) a group of patients with anemias of unknown origin, they were given the copper and iron and the rate studied at which the red blood cells and the hemoglobin returned to normal, (2) a group of patients with general paresis, they were given therapeutic malaria but no copper and iron, and the progression and regression of the resulting anemia carefully evaluated, (3) a group of general paresies who were given malaria therapy and at the same time 32 mg of ferrous iron until their anemia had disappeared.

#### RESULTS

Eleven adult individuals with varying grades of secondary anemia were given the copper and iron capsules as described. The effect of this treatment upon the anemia of the group is shown in Chart 1.

Fourteen adult general paresies were given malaria as a therapeutic measure. The effect of the malaria on red cell counts and hemoglobin of the group is shown in Chart 1.

Thirty one adult general paresies were inoculated with therapeutic malaria, and the iron treatment was started at once. The effect of the iron on the anemia of the group is shown in Chart 1.

Thirty seven adult general paresies were inoculated with therapeutic malaria, and the copper and iron treatment started at once. The effect of the combination of these two drugs on the resulting anemia of the group is shown in Chart 1.

A survey of this material brings out the following facts.

*Group 1 Secondary Anemia of Unknown Origin*—In this group of cases, 8 responded to the copper and iron with increases in red blood cell counts of from 560,000 to 1,070,000 over a period of from two to fourteen weeks. Eight responded with increases in hemoglobin of from 10 to 20 per cent over a period of two to fourteen weeks. Usually the increases in the hemoglobin and red blood cells accompanied each other, although the increases were not all proportionate. The copper and iron in the quantity used, therefore, does

\*The copper and iron preparation used was Copperin A manufactured by Myron L. Walker Inc.



increase very definitely the percentage of hemoglobin and number of red blood cells in secondary anemia. It is felt that the ability of these two chemicals to decrease a secondary anemia being established for this study, any differences observed in groups 2, 3, and 4, are probably due to copper and iron.

*Group 2. Induced Secondary Anemia in Malaria-Treated General Paretics but Untreated With Copper and Iron.*—In this group the hemoglobin reached its lowest point in four to eight weeks after the first temperature elevation. In one case the lowest hemoglobin was obtained one week before the malaria was terminated; in 2 it was reached in the same week the malaria was terminated; and in 11 cases the lowest hemoglobin was reached one to five weeks after the malaria had been terminated. The time required for the hemoglobin to reach a concentration of 80 per cent from the termination of the malaria was four to eleven weeks. The lowest hemoglobin percentage in the cases ranged from 36 to 58 per cent. The red blood cell count reached its lowest point in three to ten weeks after the first temperature elevation. In one case the lowest red blood cell count was obtained one week before the malaria was terminated; in 3 it was obtained on the same week the malaria was terminated; and in 10 cases the lowest red blood cell count was reached one to six weeks after the malaria was terminated. The time required for the red blood cells to reach their highest concentration from their lowest concentration was four to eight weeks. The time required for the red blood cells to reach their greatest concentration from the termination of the malaria was four to eleven weeks. The lowest red blood cell concentration in the group was 1.98 to 3.51 millions. The changes in the red blood cell concentration roughly paralleled the changes in the hemoglobin percentage.

*Group 3. Induced Secondary Anemia of Malaria-Inoculated General Paretics Treated With Iron.*—In this group the hemoglobin reached its lowest point in three to eight weeks after the first temperature elevation. In 2 cases the lowest hemoglobin was obtained one week before the malaria was terminated; in 5 it was reached on the same week the malaria was ended; and in 24 cases the lowest hemoglobin was reached one to three weeks after the malaria was terminated. The time required for the hemoglobin to reach a concentration of 80 per cent from its lowest concentration was one to nine weeks. The time required for the hemoglobin to reach 80 per cent from the termination of the malaria was one to ten weeks. The lowest hemoglobin percentages in the cases ranged from 37 to 68 per cent. The red blood cell count reached its lowest point in three to eight weeks after the first temperature elevation. In 4 cases the lowest red blood cell count was obtained one week before the malaria was terminated; in 9 it was obtained in the same week the malaria was ended; and in 18 cases the lowest red blood cell count was reached one to four weeks after the malaria was terminated. The time required for the red blood cell count to reach its highest concentration from its lowest concentration was one to nine weeks. The time required for the red blood cell count to reach its greatest concentration from the termination of the malaria was one to ten weeks. The

lowest red blood cell concentration in the group was 2 to 3 million. The changes in the red blood cell concentration roughly paralleled the changes in hemoglobin percentage.

*Group 4 Induced Secondary Anemia of Malaria Inoculated General Paretics Treated With Copper and Iron*—In this group the hemoglobin reached its lowest point in one to five weeks after the first temperature elevation. In 4 cases the lowest hemoglobin was obtained one week before the malaria was terminated, and in 33 it was reached one to three weeks after the malaria was terminated. The time required for the hemoglobin to reach a concentration of 80 per cent from the lowest concentration was 0 to five weeks. The time required for the hemoglobin to reach 80 per cent from the termination of the malaria was one to three weeks. The lowest hemoglobin percentages in the cases ranged from 47 to 77 per cent. The red blood cell count reached its lowest point in one to four weeks after the first temperature elevation. In 13 cases the lowest red blood cell count was obtained one week before the malaria was terminated, in 4 it was obtained in the same week the malaria was ended, and in 20 cases the lowest red blood cell count was reached one to two weeks after the malaria was terminated. The time required for the red blood cell count to reach its highest concentration from its lowest concentration was one to five weeks. The time required for the red blood cell count to reach its greatest concentration from the termination of the malaria was one to five weeks. The lowest red blood cell concentration in the group was 2.5 to 4 million. The changes in the red blood cell concentration roughly paralleled the changes in hemoglobin percentage. There was apparently no relationship between the number of chills induced by the malaria and the changes in the hemoglobin and red blood cell counts.

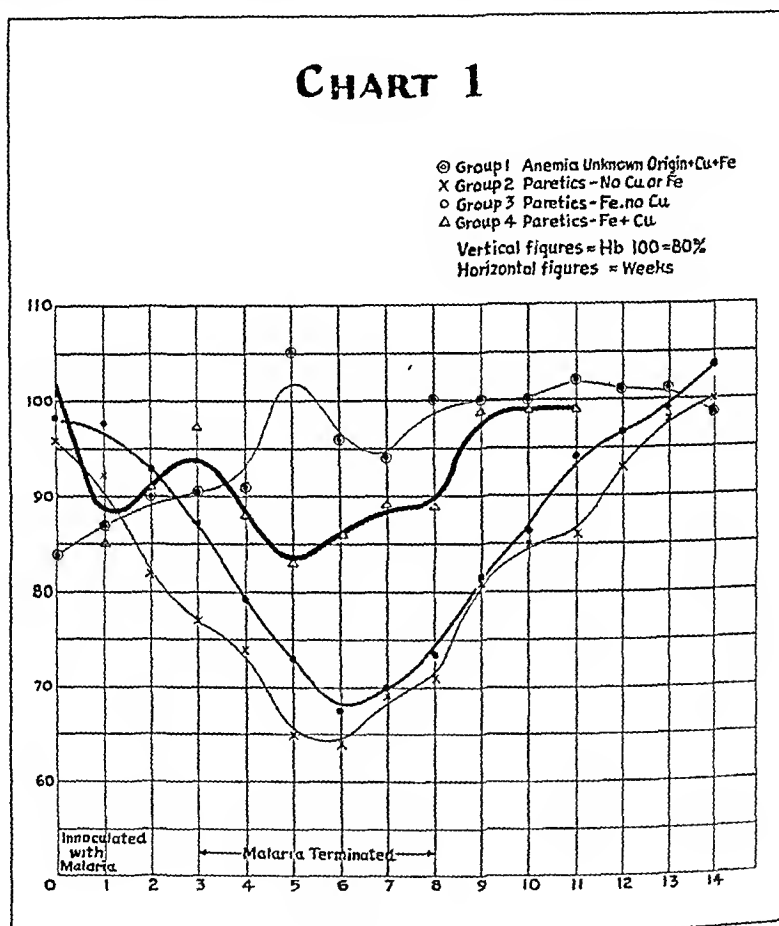
Chart 1 illustrates the average trend of the hemoglobin values in groups 1, 2, 3, and 4. The red cell curve so closely paralleled the hemoglobin curve that this latter is not presented.

Chart 2 illustrates graphically the essential facts presented in groups 1, 2, 3, and 4.

#### DISCUSSION

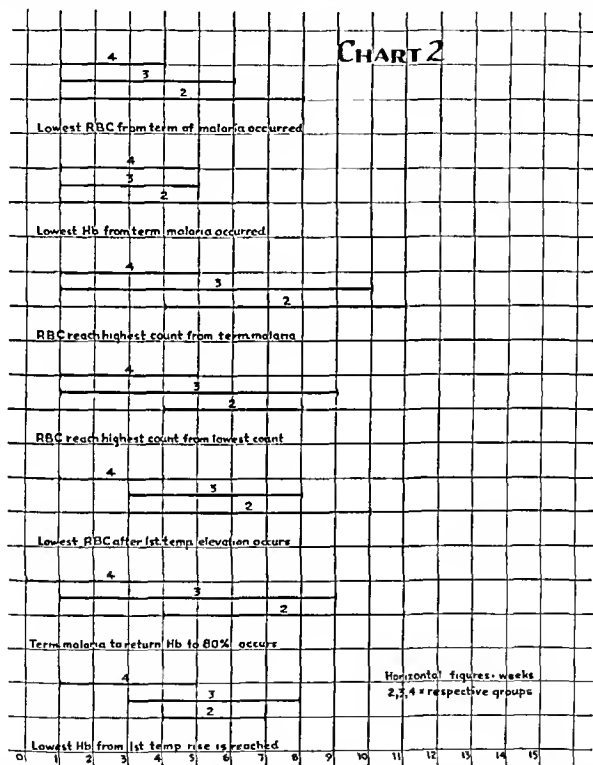
A general survey of this study would tend to establish in our cases the following facts: (1) The administration of copper and iron does stimulate hemoglobin and red blood cell formation. This is well illustrated in the cases of group 1. In these cases the combination increased the hemoglobin and red blood cells quite consistently in most instances. (2) In malaria treated general paretics (group 2) the hemoglobin and red blood cells were both depressed, and increased only after the termination of the malaria. This return to normal was a slow process, often requiring many weeks. (3) In malaria treated general paretics (group 3) when ferrous iron was administered regularly from the time of the malarial inoculation to the return of the blood picture to normal, the average curves of depression and recuperation of the blood picture were approximately the same as those in group 2.

When, however, the lowest hemoglobin and red blood cell values and their distances from the first temperature elevation were studied, it was found that there was a definite shift of some of these low values away from the first temperature elevations. And when the distances from the lowest hemoglobin and red blood cell counts to the last (or first normal) hemoglobin and red blood cell counts were studied, it was observed that the distance (time) of hemoglobin and red cell recovery was lessened. The secondary anemias of group 3, moreover, were not as profound as those of group 2. (4) In malaria-treated general paretics (group 4) when copper and iron were regularly admin-



istered from the time of the malarial inoculation to the return of the blood picture to normal, the average curve of depression and recuperation of the blood picture as illustrated in the hemoglobin curve in Chart 1 was quite different from those of groups 2 and 3. The curve dropped slower, and before it had dropped half of the distance of those of group 2 or 3, it began to rise again and reached normal far more rapidly than those of either group 2 or 3. As is shown in Chart 2 the time from the first temperature elevation to the lowest hemoglobin and red cell counts was usually shorter, and the return to normal was generally more rapid. This lessened severity of the anemia coupled with the fact that it was more difficult for the malaria to produce the

anemia and the fact that the red blood cell counts and hemoglobin were rapidly restored to normal by means of copper and iron, would indicate that these chemicals are of value in combating the anemia incurred by malaria. There was no evidence that they interfered in any way with the therapeutic benefit derived from the malaria; nor did these drugs interfere with the temperature rises in any way. The copper and iron, furthermore, did not interfere with the action of tryparsanide when this latter drug was administered.



We also feel that they can be used with impunity in dosages much larger than the ones used in this study. It is highly probable that such larger dosages would be more satisfactory than the smaller ones we have used.

Our work indicates, moreover, that the reticulo-endothelial system of man can be stimulated to such an extent that it can minimize the devastation wrought in the blood stream by malaria. It also indicates that the malaria did not materially affect the reticulo-endothelial system of the cases studied and thereby intensify the anemia produced by direct blood destruction.

## SUMMARY

A study of the effects of copper and iron on the secondary anemia of therapeutic malaria in general paresis is presented. The results would indicate that these two drugs are of value in lessening the severity and duration of this type of secondary anemia.

## REFERENCES

1. Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A.: Iron in Nutrition, Copper as a Supplement to Iron for Hemoglobin Building in Rat, *J. Biol. Chem.* 77: 979, 1928.
2. Elvehjem, C. A., Steenbock, H., and Hart, E. B.: Is Copper a Constituent of Hemoglobin Molecule? Distribution of Copper in Blood, *Ibid.* 83: 21, 1929.
3. Elvehjem, C. A., and Sherman, W. C.: Action of Copper in Iron Metabolism, *Ibid.* 98: 309, 1932.

## STUDIES IN BONE MARROW\*

ABRAHAM S. GORDON, M.D., F.A.C.P., BROOKLYN, N. Y.

THE bone marrow is one of the mysterious and intriguing organs or tissues in the human body. In its significance it may be compared to the central nervous system. There are some similarities and also striking differences between these two tissues. Nature must have considered both very important when it protected them with a hard, resistant, skeletal system—the only two tissues in the body which are enclosed within a bony casing. Both tissues show a quick response to any injury or danger involving the general organism. Oftentimes the bone marrow will show changes, as evidenced by the circulation, long before any other evidence is apparent. On the other hand, while the central nervous system is the most highly specialized tissue in the body, the bone marrow is the most embryonic tissue, and is the only tissue in the body which is persistent as such throughout life, with the exception of such elements as the splenic pulp and several other minor foci of the reticulo-endothelial system. The bone marrow in contradistinction to all fixed tissues, is a potentially wandering tissue in that its elements, which are the product of its activity and proliferation, are the future wandering cells, scattered throughout the circulation and tissue spaces in all parts of the body.

Up to a very short time ago only tissue pathology of the hematopoietic system has been studied intensively, cellular pathology having been considerably neglected since the days of Virchow. To Sabin and her co-workers is due a good deal of the credit for the revival and development of the supravitral method of study, this method having been dormant for many years since its inception at the beginning of this century. Her work accomplished in this field is monumental and has helped greatly in the revival of the study of cellular pathology, about which so much is heard now, especially from the standpoint of the reticulo-endothelial system and hematopoiesis. It is from this latter standpoint that this work was undertaken. The elements of the marrow rather than its histologic

\*From the Department of Pathology, the Jewish Hospital, Brooklyn.  
Received for publication, April 15, 1938.

structure was the aim of this study, and the investigation consisted of a study of the quantities of the different types of cells per unit volume of bone marrow, the relative proportion of the various elements to one another, and the character of the different cells in their various stages of differentiation. Beginning with the different types of cells as they are found in the circulation, classified and unclassified, which types are more common and abundant in the bone marrow where earlier forms are discernible, it was hoped to follow these elements in their various stages to their origin in the forms of the primordial blood cells.

The difficulties encountered in the study of the bone marrow may best be summarized by quoting some workers in this field (Doan and Zeffas, *J. Exper. Med.* 46, 531, 1927): "Any attempt at a quantitative estimation of the various cellular elements making up the bone marrow must, in the very nature of the survey, always be open to many questionings. The limitations inherent in any technique used, the fallacies inseparable in trying to draw any general deductions from even 1,000 or 2,000 cells counted out of the multiple millions present in any functioning bone marrow, the much debated questions of identification and classification of immature forms, the many factors known and unknown affecting hemopoiesis in any particular individual—all of these and more make it necessary to be conservative in the drawing of deductions from any one limited series of observations or in trying to compare the figures obtained from different investigators."

Accordingly, this study is presented merely as interesting data, observations, and method of procedure. Use was made of material as it became available at the post mortem table, without reference to any particular type of disease or group of cases. The investigation included all ages and ranged from birth to extreme old age, with a variety of diagnoses in every stage of life.

The same principle was used as that for counting the cells in the circulation, an acetic acid solution for the nucleated elements, and a citrate solution for the red cells as well as all elements. The method may be termed "The Dilution Method." In all cases only rib marrow was used for the quantitative estimation. In most cases one part of marrow was diluted in nine parts of 3 per cent sodium citrate solution, and 2 per cent acetic acid solution, thus giving a suitable dilution for counting, and a convenient factor for calculation. Some cases required a modification of this proportion.

#### METHOD OF PROCEDURE

Special tubes, 7.5 cm. in length by 7.5 mm. in diameter, were used. These tubes were graduated in tenths of a cubic centimeter. One of these tubes contained 0.9 cc. of 2 per cent acetic acid solution, and another tube 0.9 cc. of 3 per cent sodium citrate solution. To each of these tubes were added 0.1 cc. of bone marrow, and the tubes were shaken gently for about fifteen minutes. The separation of the marrow fat and its rising to the surface of the solution not only did not interfere with the counting process, but aided it considerably in that the counting chamber was almost free from fat particles. The few cells which were enmeshed in the fat did not make any appreciable difference in the results. The dilutions were then drawn up into the proper blood counting pipettes to the 0.5 or 1 mark, as the case required, depending upon the con-



Fig. 1.—Bone marrow smear from a case of myeloid leucemia. Note the proportion of the myeloid, metamyeloid, and the mature polymorphonuclear cells.

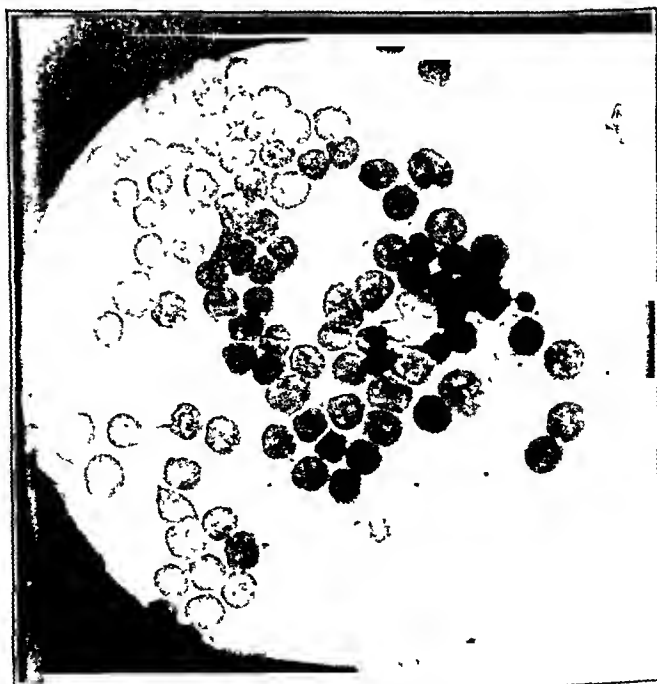


Fig. 2.—Bone marrow smear from a case of lymphoid leucemia. Note the proportion to the myeloid cells.



FIG 3—Bone marrow smear from a case of myeloblastic leucemia

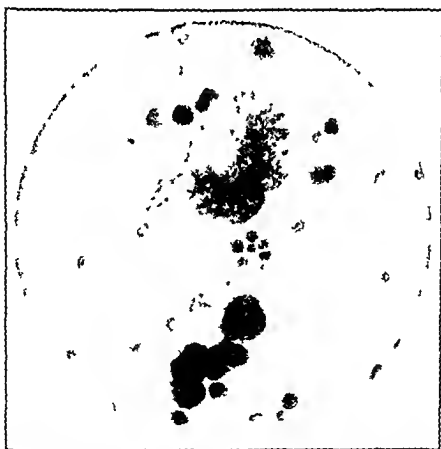


FIG 4—Smear of bone marrow showing megakaryocytes. Note their comparison with the other bone marrow elements



sistency of the marrow, and the rest of the pipette filled with the appropriate solution. The pipettes were again shaken for about ten minutes, and the counts made in the Neubauer hemocytometer. The calculations were made accordingly.

In all cases the total number of all cells and the total number of nucleated cells were counted separately from the different pipettes in different counting chambers. Then the total number of red cells and the total number of nucleated cells were counted separately in the same chamber of the citrate solution, and the two independent figures for the nucleated cells from the two different solutions were compared. In no case were the final figures recorded unless the two separate figures corresponded, allowance having been made for the average technical error. All nucleated cells were counted in one group, comprising all myeloid cells; all nucleated red cells, including normoblasts, megaloblasts, and

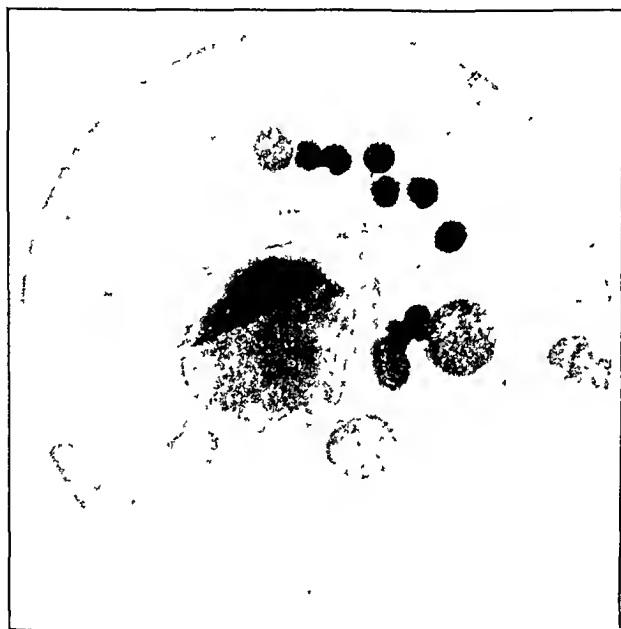


Fig. 5.—Another type of megakaryocyte with a smooth and vacuolated cytoplasm.

still earlier forms of the erythroid series; all lymphoid cells, as well as endothelial cells and all forms of unclassified cells. In some cases, when indicated, the megakaryocytes were counted separately. The further subdivision of these cells becomes a problem for differential study of the stained smear, for attempts were not made to differentiate the various types of cells in the counting chamber. The results obtained are presented in tabular form, giving the sex and age of the patient, the clinical and pathologic diagnosis, the number of red blood cells and nucleated blood cells per cubic millimeter of blood as found in the circulation a short time before death, the red blood cells and nucleated cells of all types as found in the bone marrow per cubic millimeter of marrow.

#### DIFFERENTIAL STUDY

The same "dilution principle" was used as that for the quantitative estimation. However, for the differential study blood serum was used for dilution. Autogenous serum, of course, is ideal, but this was not always procurable.

Therefore, the blood group of the individual was determined from the marrow, and corresponding serum was used for dilution. The proportion of serum to bone marrow cannot be fixed for use in all cases, as each marrow requires an amount commensurable with its own physical characteristics, and the proper quantity to be used must be judged from experience. Sometimes several mixtures must be made before obtaining a proportion which would give the proper smears. The Romanowsky dyes were used for staining. In some cases, such as myeloblastic leucemia, lymphoid leucemia, agranulocytic angina, aplastic anemia, or any other forms of severe anemia of unknown etiology, the results were counterchecked with the supravitral stain and the oxydase stain. Satisfactory results were obtained in most cases, except those in which the autopsy material was not fresh, or when the autopsy was done a long time after death.

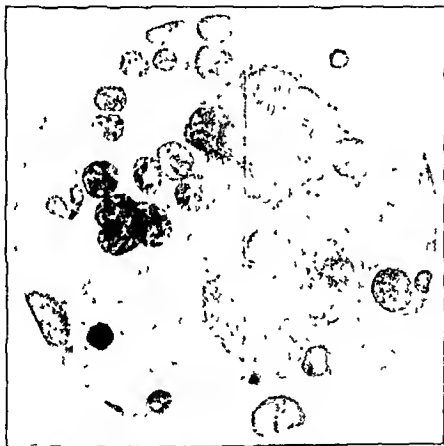


Fig. 6—Three megakaryocytes in one field. This smear was made from a bone marrow of a case of myeloid leucemia.

Such unsatisfactory material was discarded after attempts to study them, and the results omitted from the record.

#### DISCUSSION

An analysis of Table I is tempting of many comments. To begin with, the proportion of nucleated cells to red blood cells in the circulation normally is about 1:700, while in the bone marrow the proportion drops considerably, reaching almost 1:1, and in many cases the nucleated elements are more abundant than the red cells. This is probably due to the fact that the red cell has a rather long life and a great storage of these elements is not necessary, while the short-lived white cell requires an abundant storage supply for a constant and rapid turnover. After studying many pathologic bone marrows, the question arose as to what is the normal cellular content of the bone marrow per unit volume. The opportunity for such observation presented itself in Case 23. A young healthy male, 28 years old, was working on a scaffold repairing the outside of a

TABLE I

NO.	SEX	AGE	DIAGNOSIS	R.B.C. IN CIRCULATION	R.B.C. IN BONE MARROW	NUCLEATED CELLS IN CIRCULATION	NUCLEATED CELLS IN BONE MARROW
1	M	2	Otitis media. Meningitis	4,020,000	2,130,000	22,000	920,000
2	F	33	Addison's disease	4,320,000	4,100,000	18,000	1,480,000
3	M	62	Acute myeloblastic leucemia	2,300,000		19,000 (3d) 3,800 (1d) 400 (5h)	220,000
4	F	34	Endocarditis. Terminal pneumonia	4,180,000	840,000	18,600	1,200,000
5	F	48	Diabetes. Terminal pneumonia	4,180,000	1,125,000	19,200	1,024,000
6	M	8	Aplastic anemia	2,120,000	110,000	750	28,000
7	M	33	Myeloid leucemia	2,850,000		96,000 (5d) 128,000 (3d) 130,000 (1d)	1,024,000
8	M	70	Cardiac decompensation	4,160,000	704,000	8,100	512,000
9	M	59	Hypernephroma	3,600,000	1,152,000	11,800	416,000
10	M	8 mo.	Sepsis	4,120,000	2,464,000	40,000	968,000
11	M	68	Aneurysm. Syphilis	4,900,000	1,072,000	10,000	512,000
12	F	45	Cardiac decompensation	4,320,000	488,000	6,500	672,000
13	M	6	Lung abscess	2,430,000	1,464,000	15,300	960,000
14	M	63	Carcinoma of pancreas	3,700,000	648,000	11,800	384,000
15	M	2	Sepsis	4,120,000	680,000	16,300	412,000
16	M	1	Syphilis. Pneumonia	3,100,000	1,160,000	33,000	1,024,000
17	M	5 mo.	Otitis media. Bronchopneumonia	6,150,000	5,792,000	9,400	288,000
18	M	3 mo.	Otitis media. Meningitis	4,460,000	1,056,000	14,800	472,000
19	M	64	Coronary sclerosis	5,440,000	558,000	7,100	604,000
20	F	52	Agranulocytic angina	4,900,000	1,120,000	1,100	224,000
21	M	45	Lung abscess	3,810,000	1,112,000	13,400	408,000
22	M	73	Adenocarcinoma of left kidney	4,350,000	1,352,000	9,500	480,000
23	M	28	Traumatic shock		1,132,000		468,000
24	M	17	Acute nephritis	4,560,000	720,000	11,400	788,000
25	M	7 mo.	Sepsis	7,550,000	2,218,000	10,650	240,000
26	M	2 wk.	Bronchopneumonia	4,660,000	2,320,000	30,600	536,000
27	F	2 mo.	Rickets. Sepsis	4,300,000	1,096,000	30,000	1,024,000
28	M	56	Polycythemia	13,000,000* 10,760,000* 5,200,000*	1,168,000	29,000 (3m) 16,000 (2m) 13,400 (3d)	512,000
29	M	50	Myocarditis. Terminal pneumonia	4,050,000	912,000	11,000	788,000
30	M		Stillbirth		2,120,000		576,000
31	M	39	Agranulocytic angina	4,440,000	672,000	200	208,000
32	M	24	Aplastic anemia	850,000	336,000	2,600	80,000
33	M	56	Lymphatic leucemia	1,664,000* 984,000*	192,000	233,600 (5d) 342,000 (1d)	1,320,000

m = months before death  
d = days before death

h = hours before death

\* = same time as nucleated cells in circulation

building. He lost his balance, slipped, and fell off the scaffold, striking the concrete sidewalk underneath. He was rushed to the hospital at once, but he died soon after reaching it. A post mortem examination was done within a half hour after the accident occurred, and the bone marrow showed a red cell count of 1,132,000, and a nucleated cell count of 468,000 per cubic millimeter. There was no history of previous illness, and the man was said to be in excellent health since childhood. Histologic studies of all organs and tissues showed no pathologic changes. The findings then are assumed to be those of a normal healthy individual. It is more interesting, therefore, to compare these findings with those seen in Case 21, with the diagnosis of lung abscesses of several months' duration following an acute pneumonia, or with the findings in Case 22, with the diagnosis of adenocarcinoma of the kidney (see also Cases 9 and 18). One probable explanation may be that whatever the original stimulus and its resultant bone marrow response, when the disease had progressed for many months or longer, either the stimulus or the mechanism of response, or both, were exhausted, and the bone marrow in time began to assume a static position in its activity. Frequently evidence in the circulation tends to corroborate this view.

In other cases, the bone marrow findings may be misleading without an adequate and detailed study from other angles. This is particularly true in some of the blood diseases. In Case 7, a case of myeloid leucemia, in which the nucleated cell count in the circulation is over 100,000 per cubic millimeter, the nucleated cell count in the bone marrow does not reflect the unusual proportion, but merely shows a count two or three times the average normal amount. However, when one takes into account the tremendous increase in the total volume of marrow, as shown by the large size of the rib, with almost a spherical diameter, and the densely packed succulent marrow, the proportion is seen to be much greater than it appears at first glance. One may also speculate at the role played by the endocrine system in the change of the skeletal structures containing the marrow. Surely the change in the size of the bony tissue must have been going on for a long time before the leucemic state made its appearance or was recognized clinically. This change in the rib structure is not seen in lymphoid leucemia, probably because of the infiltrative rather than proliferative character of this type of leucemia in the bone marrow, and the displacement of the red cell elements by this infiltration. Another point of interest is the minimum or lowest number of nucleated cells in the marrow seen in such cases as aplastic anemia, agranulocytic angina, or certain cases of myeloblastic leucemia. When the bone marrow reaches a certain low level, no nucleated cells will trickle through into the circulation. These and many other phases of bone marrow activity tempt one to speculate as to the interpretation of these findings in the hope of correlating them with clinical applicability.

#### SUMMARY

1 A method is presented for the quantitative estimation of the cellular elements of the bone marrow and for differential study.

2 By this method results were obtained which promise an approach to the study of bone marrow cytology from new angles.

# LETHAL DIFFERENCES BETWEEN PANCREATIC EXTRACT (WOLFFE) AND ACETYL-BETA-METHYLCHOLINE IN ATROPINIZED ANIMALS\*

VICTOR A. DIGILIO, M.D., JOSEPH A. PESCATORE, M.D., AND  
HAROLD E. GOLDBERG, M.D., PHILADELPHIA, PA.

EXTRACTS of animal tissues have long been known to contain impurities such as histamine and choline. Objections have been raised against pancreatic extracts on these grounds. As a matter of fact, it has been intimated that the therapeutic efficacy of these extracts may be due to the impurities.

Wolffe and Munch<sup>1</sup> showed that Norit-treated pancreatic extracts retained their potency and did not lose their heart-blocking properties. Moreover, they added known quantities of histamine to the solutions and were able to recover practically 100 per cent by this method. It has also been shown<sup>2</sup> that atropine prevents the depressor action of acetylcholine, but fails to prevent the depressor action of pancreatic extract. In spite of this, criticism still exists. Since both substances when administered in adequate dosage cause heart block in animals, and since atropine is pharmacologically antagonistic to acetyl- $\beta$ -methylcholine, another method presents itself for the detection of any differences in action between pancreatic extract (Wolffe) and acetyl- $\beta$ -methylcholine. If the former substance owes its activity to the presence of choline impurity, it should be expected to act in a fashion similar to the action of any pure choline substance.

## METHOD

Choline impurities in pancreatic extract are an unknown quantity. In order to compare these substances in animals it was obviously necessary to ascertain the smallest amount of each substance which would consistently cause heart block and death in white mice, of a known strain, weighing between 24.3 and 25.8 gm. each. It was found that 1 c.c. of pancreatic extract made according to Wolffe<sup>3</sup> and standardized according to the published method<sup>4</sup> (so that 1 c.c. of extract possessed 20 gamma epinephrine neutralizing units) caused death by intraperitoneal injection within five to nine minutes in 9 out of 10 animals (the last animal died of intra-abdominal hemorrhage). It was observed that the animals consistently developed heart block, but that respiration ceased while occasional ventricular systole could still be registered electrocardiographically for several seconds afterward. It was likewise determined that 7 mg. of acetyl- $\beta$ -methylcholine (7 mg. per c.c.) by intraperitoneal injection caused heart block and death in 10 animals. In this group

\*From the Temple University Hospital.  
Received for publication, May 14, 1938.

the heartbeat and respirations ceased simultaneously within six to nine minutes. Smaller doses of either substance caused lesser degrees of cardiac slowing but seldom death.

A group of 20 white mice of the same strain and weight as the above were divided into subgroups of 5 each. All the animals were kept under the same conditions of temperature, feeding time, etc. All experimental procedures were carried out at the same time of the day, and each animal was

#### Protocol I

##### Lethal Dosage of Acetyl $\beta$ Methylcholine Chloride\*

Mouse No 273	male—25.3 gm		
	2.19—7.0 mg acetyl $\beta$ methylcholine	8	minutes
	2.27—animal dead		
Mouse No 275	male—24.5 gm		
	3.05—7.0 mg acetyl $\beta$ methylcholine	64	minutes
	3.11—animal dead		
Mouse No 276	male—25.1 gm		
	3.30—7.0 mg acetyl $\beta$ methylcholine	74	minutes
	3.37—death		
Mouse No 277	male—25.6 gm		
	3.41—7.0 mg acetyl $\beta$ methylcholine	9	minutes
	3.50—death		
Mouse No 278	male—24.4 gm		
	3.53—7.0 mg acetyl $\beta$ methylcholine	8	minutes
	3.59—death		

\* Acetyl  $\beta$  methylcholine was supplied by Merck & Co. as the chloride.

#### Protocol II

##### Effect of Pancreatic Extract on Mice

Mouse No 285	male—25.5 gm		
	3.12—1 cc pancreatic extract (Wolffe)		
	3.15—animal breathing slowly and irregularly		
	3.17—animal dead		
Mouse No 286	male—24.7 gm		
	3.19—1 cc pancreatic extract (Wolffe)		
	3.27—animal dead		
Mouse No 287	male—24.3 gm		
	3.30—1 cc pancreatic extract (Wolffe)		
	3.35—animal dead		
Mouse No 288	male—25.4 gm		
	3.36—1 cc pancreatic extract (Wolffe)		
	3.43—animal dead		
Mouse No 289	male—25.1 gm		
	3.46—1 cc pancreatic extract (Wolffe)		
	3.52—animal dead		

NOTE. All mice Nos 285-289 died after having a convulsive spasm during which their limbs became rigid, bending toward each other, the body as a whole becoming very taut and the tail rising almost perpendicularly. After a few seconds in this position the body relaxed, the tail came down, and the animal ceased breathing.

weighed at the beginning of the experiment. Animals under 24.3 gm or over 25.8 gm were discarded. The subgroups were arranged as follows:

- Group 1 received acetyl  $\beta$  methylcholine
- Group 2 received pancreatic extract
- Group 3 received acetyl  $\beta$  methylcholine after atropinization
- Group 4 received pancreatic extract after atropinization

All injections were made by the intraperitoneal route. One cubic centimeter volumes were employed in each injection, so that 1 cc of acetyl  $\beta$ -

# LETHAL DIFFERENCES BETWEEN PANCREATIC EXTRACT (WOLFFE) AND ACETYL-BETA-METHYLCHOLINE IN ATROPINIZED ANIMALS\*

VICTOR A. DIGILIO, M.D., JOSEPH A. PESCATORE, M.D., AND  
HAROLD E. GOLDBERG, M.D., PHILADELPHIA, PA.

EXTRACTS of animal tissues have long been known to contain impurities such as histamine and choline. Objections have been raised against pancreatic extracts on these grounds. As a matter of fact, it has been intimated that the therapeutic efficacy of these extracts may be due to the impurities.

Wolffe and Munch<sup>1</sup> showed that Norit-treated pancreatic extracts retained their potency and did not lose their heart-blocking properties. Moreover, they added known quantities of histamine to the solutions and were able to recover practically 100 per cent by this method. It has also been shown<sup>2</sup> that atropine prevents the depressor action of acetylcholine, but fails to prevent the depressor action of pancreatic extract. In spite of this, criticism still exists. Since both substances when administered in adequate dosage cause heart block in animals, and since atropine is pharmacologically antagonistic to acetyl- $\beta$ -methylcholine, another method presents itself for the detection of any differences in action between pancreatic extract (Wolffe) and acetyl- $\beta$ -methylcholine. If the former substance owes its activity to the presence of choline impurity, it should be expected to act in a fashion similar to the action of any pure choline substance.

## METHOD

Choline impurities in pancreatic extract are an unknown quantity. In order to compare these substances in animals it was obviously necessary to ascertain the smallest amount of each substance which would consistently cause heart block and death in white mice, of a known strain, weighing between 24.3 and 25.8 gm. each. It was found that 1 c.c. of pancreatic extract made according to Wolffe<sup>3</sup> and standardized according to the published method<sup>4</sup> (so that 1 c.c. of extract possessed 20 gamma epinephrine neutralizing units) caused death by intraperitoneal injection within five to nine minutes in 9 out of 10 animals (the last animal died of intra-abdominal hemorrhage). It was observed that the animals consistently developed heart block, but that respiration ceased while occasional ventricular systole could still be registered electrocardiographically for several seconds afterward. It was likewise determined that 7 mg. of acetyl- $\beta$ -methylcholine (7 mg. per c.c.) by intraperitoneal injection caused heart block and death in 10 animals. In this group

\*From the Temple University Hospital.  
Received for publication, May 14, 1938.

methylcholine solution contained 7 mg. of drug and 1 c.c. of Wolffe extract contained 20 epinephrine neutralizing units. In each instance electrocardiograms were taken employing hypodermic needles embedded into the limb muscle of the animals and connected to the Lead II wires of an electrocardiograph

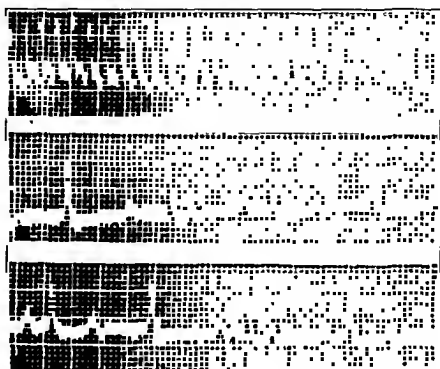


Fig. 1.—Acetyl- $\beta$ -methylcholine control. *a*, Control tracing, *b*, four and one-half minutes after injection of acetyl- $\beta$ -methylcholine, showing heart block, *c*, heart block continued and death of the animal occurred shortly afterwards.

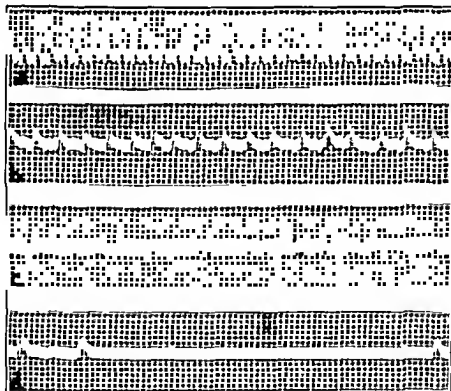


Fig. 2.—Pancreatic extract control. *a*, Control tracing, *b*, slowing of heart rate with beginning inversion of initial ventricular complex, *c*, complete heart block and complete inversion of both ventricular complexes, *d*, death nine and one-half minutes after injection and initial ventricular complex became upright again.

(G. E. portable; right forelimb and left hindlimb) by conductor clips. Tracings were taken before and at intervals after injecting the animals. Atropine sulfate in doses of 0.013 mg. per c.c. was injected into animals to be atropinized, and fifteen minutes allowed for complete absorption before administering the



- (b) The lethal effects of acetyl- $\beta$ -methylcholine were prevented by atropinization.
- (c) Atropine did not prevent the complete heart block which pancreatic extract (Wolffe) consistently caused in the controls.
- (d) Although bradycardia occurred in the atropinized animals which received acetyl- $\beta$ -methylcholine, it was not as severe in degree as the complete A-V dissociation seen in the controls.

2. Electrocardiographic differences between the control groups were obviously definite.

- (a) The pancreatic animals exhibited inversion of the initial ventricular complexes and ectopic ventricular arrhythmias not demonstrated in the tracings of the animals receiving acetyl- $\beta$ -methylcholine.

3. Since the dosage of pancreatic extract (Wolffe) employed in these experiments is out of all proportion per body weight to the largest doses used in human beings, the above observations cannot be used as an index of toxicity.

#### CONCLUSION

The above findings suggest fundamental differences of action between acetyl- $\beta$ -methylcholine and pancreatic extract (Wolffe). The magnitude of this disparity warrants the statement that the choline substances present in pancreatic extract (Wolffe) do not account for the action of this extract under the conditions of this experiment, unless such substances (choline) possess properties heretofore not attributed to or demonstrated by choline derivatives.

#### REFERENCES

1. Wolffe, J. B., and Munch, J. C.: The Effect of Norit on the Heart-Bleeding Constituent of Tissue Extract No. 568 ("Desympatone"), *Arch. internat. de pharmacodyn. et de therap.* 44: 387, 1933.
2. Wolffe, J. B., and Munch, J. C.: Angina Pectoris, Its Treatment With Insulin Free Pancreatic Extract ("Desympatone"), *Delaware State M. J.* 7: 123, 1933.
3. Wolffe, J. B., Findlay, D., and Dessen, E.: Treatment of Angina Pectoris With a Tissue Vasodilator Extract—Preliminary Report, *Ann. Int. Med.* 5: 625, 1931.
4. Wolffe, J. B., and Munch, J. C.: Pancreatic Hormones: Extracted After Removal of Insulin, *Summaries of Communications, 14th International Physiological Congress in Rome, Italy, August, 1932.*
5. Wolffe, J. B., Munch, J. C., Rabinowitz, H. M., and Digilio, V. A.: Desympatone—A Fraction of Insulin-Free Pancreatic Extract, *Summaries of Communications of the 15th International Physiological Congress, Leningrad, Moscow, August, 1935.*

## CORONARY ARTERY THROMBOSIS\*

F JANNY SMITH, M D, BEN E GOODRICH M D, AND ROBERT J NEEDLES, M D  
DETROIT, MICH

A RECENT survey of the cases of acute coronary artery thrombosis at the Henry Ford Hospital has been carried out in an attempt to evaluate the experiences of the past in the light of present knowledge. Since the installation of our electrocardiographic instrument in 1923, there have been 159 cases of undoubted coronary occlusion in this hospital. One hundred eleven patients were successfully traced, and of these 83 were dead. 46 of the latter number had been examined post mortem. The reclassification of the cases was done with the plan of Wilson and co-workers, dividing them by means of electrocardiographic changes into anterior, posterior and indeterminate groups. The material at hand has been grouped as in Table I.

TABLE I  
CASES STUDIED

Total cases	159
Studied with EKG	146
Cases traced to date	111
Cases traced—still living	28
Cases traced—died	83
Autopsied	46
Cases dead in first year	60 (54 per cent)

This material was critically selected from perhaps twice that number of cases indexed as coronary thrombosis. Those which, for one reason or another, failed to answer a careful inquiry were cast out, and the resulting group is felt to be that of proved cases only.

Pertinent factors, such as age, sex, race, and occupation, and of the clinical aspects, such as the number of cases showing fever, leucocytosis, pericardial friction rub, arrhythmias, etc., were not different from previously published series of cases. The monograph of Levine<sup>2</sup> and the recent book edited by Levy<sup>3</sup> contain adequate statistical information regarding these factors. In the present series the youngest case was 29 and the oldest 79 years, while the average age of the entire group was 55.8 years. The decade of greatest incidence was from 60 to 69 years. The sex ratio was 5.36 males to 1 female. In the absence of syphilis the disease was not found to occur in colored persons in this series. Definite obesity was more frequently present than absent. No equitable classification of occupation could be made from a survey of our records, because the factors of pressure, emotional tension, and nervous stimuli are so variable.

\*From the Department of Medicine, Henry Ford Hospital, Detroit.

Read in abstract before the Central Society for Clinical Research, Chicago, November 1937.

Received for publication April 16, 1938.

in all types of activity. It is interesting to record that only 47.7 per cent presented a story of previous angina of effort. Eighty-one and seven-tenths per cent of the group gave a history of crushing, constricting, viselike pain. In 77 per cent of all cases there was continuous pain of longer than four hours' duration. The number would undoubtedly have been higher, except that many of the patients were relieved by hypodermic medication. Vomiting occurred in more than one-fourth of the patients, and prostration was a frequent manifestation.

The clinical observations on patients suffering from acute coronary artery thrombosis characteristically include fever, leucocytosis, and fall in blood pressure. In somewhat lessened frequency are found gallop rhythm, cardiac arrhythmias, and pericardial friction rub. The changes in the blood sedimentation rate appeared to persist longer than the other evidence of acute tissue damage.

In this report, however, the primary interest is in the accuracy of the electrocardiographic information as to the presence of myocardial infarction and the localization of the infarction if present. A similar study has been previously published by Sprague and Orgain.<sup>7</sup> The question of electrocardiographic localization of myocardial infarction was given its initial clinical impetus by Barnes and Whitten,<sup>4</sup> their work being based, in part, on a previous contribution by Parkinson and Bedford.<sup>5</sup> The latter authors had differentiated electrocardiograms of the  $T_1$  and  $T_3$  types, while Barnes and Whitten demonstrated the relationship of  $T_1$  curves to anterior and  $T_3$  to posterior infarction. Later Wilson and co-workers<sup>1</sup> noted the frequency of Q-wave prominence in the two major types of infarction, and elaborated this concept into electrocardiograms of the  $Q_1T_1$  and  $Q_3T_3$  types. In the present study the standard three lead electrocardiograms were styled  $Q_1T_1$  type, indicating an anterior infarction by left coronary artery thrombosis, or  $Q_3T_3$  indicating a posterior infarction by right coronary artery thrombosis.

Fig. 1 outlines the plan ordinarily used in this clinic to differentiate the various locations of infarction. This figure represents the presence of the primary S.T.<sub>1</sub> elevation with subsequent evolution into  $Q_1T_1$  contour suggesting anterior infarction. Infarction in the posterior wall is anticipated when the primary S.T. elevation occurs in Lead III.

On analysis of our electrocardiograms, some were clearly examples of anterior infarction, some were clearly posterior, while a third group, just as certainly made up of acute coronary artery thrombosis, did not fit into the two clearly differentiated groups. These have been styled indeterminate types of coronary occlusion. Some of them show only small complexes; others have various conduction disturbances, such as auricular flutter, which vitiate any attempt to localize the area of damaged heart muscle.

Of the 146 cases classified according to the above plan, 111 have been traced. Eighty-three of these are dead and 28 have survived. In Fig. 3 are seen the survival periods of the cases whose histories are finished, that is, the dead cases.

It will be noted that the greatest mortality was in the first few months, regardless of the type of electrocardiographic abnormality. Thus, in Fig. 2 it will be noted that of the anterior group, 51 per cent were dead in the first year,

while 45 per cent of the posterior group had a similar fate. The indeterminate group had a somewhat higher first year mortality (70 per cent), perhaps because many of them were of the small complex, or conduction disturbance type. It is noteworthy that in the 83 patients who died, prognostic implications of electrocardiograms of the  $Q_1T_1$  and of the  $Q_3T_3$  type are very similar. This is in op

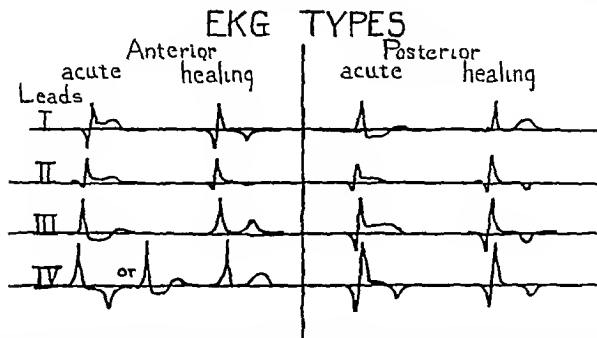


Fig 1—Chart of usual electrocardiographic changes met with in the anterior and posterior types of coronary artery thrombosis illustrating basis of differentiation made in this study. Axial leads in anterior infarction show major changes in Lead I tending to the  $Q_1T_1$  type of curve. Posterior infarction is indicated by major changes in Lead III tending toward the  $Q_3T_3$  type. Alterations in Lead IV prevail in anterior infarction.

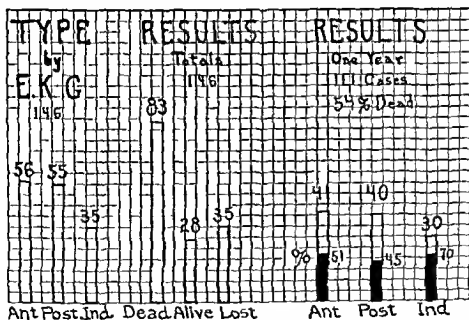


Fig 2—Illustrating the relative proportion of anterior posterior and indeterminate forms of coronary artery thrombosis as indicated by EKG changes. Also shown are the mortality rates in the first year for the three classes of thrombosis. It will be noted that anterior and posterior infarction carry almost the same first year mortality.

position to the view expressed by Wood and associates,<sup>6</sup> who felt that the immediate and future prognosis of the posterior, or  $Q_3T_3$ , group was less grave. In our experience the posterior group demands the same conservatism in prognosis as does the anterior group.

The preceding discussion as to electrocardiographic differentiation and survival period has dealt largely with the clinical aspects of the condition. The result of this survey forces the conclusion that in a few cases the electrocardiographic

changes and the autopsy findings will not be in strict agreement. Thus, of 16 fresh single infarctions, localization by the standard three lead electrocardiograms was accurate in only 5 instances. It gave definitely misleading information in 3 cases. Of the 8 doubtful cases, all having anterior infarctions, 1 showed no detectable abnormality, 4 were indeterminate, and 3 suggested posterior infarction. Hence, localization of cardiac infarction, using only the standard 3 leads, is frequently impossible and may lead to false conclusions.\*

With this thought in mind, 46 autopsied cases of coronary thrombosis have been studied (Table II). Of these, 31 were studied with one or more electrocardiograms. Since it was realized that cases showing marked coronary artery

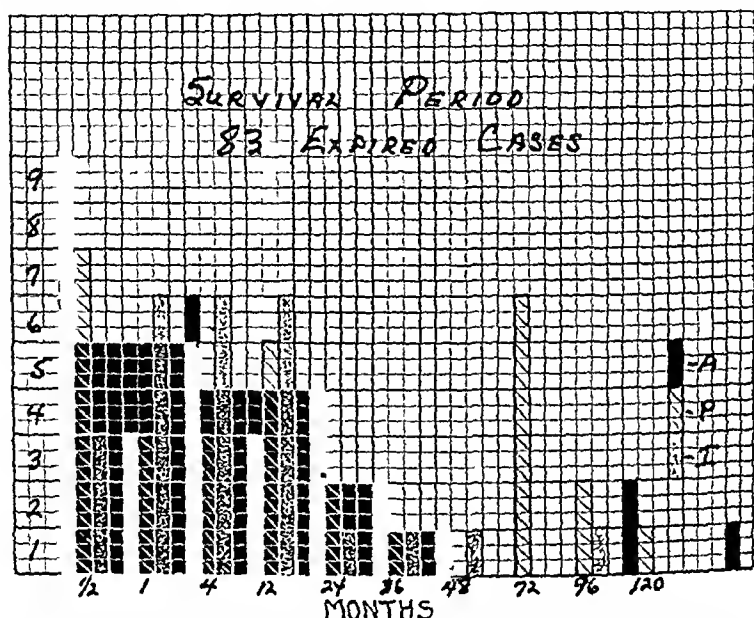


Fig. 3.—Illustrating the survival period of 83 dead cases. A, the anterior occlusions as indicated by EKG changes; P, the posterior; and I, those with changes of indeterminate origin.

sclerosis and multiple infarctions might show confusing pictures in the electrocardiogram,<sup>8</sup> the cases were divided into those with single fresh infarcts and those with associated additional pathologic changes. Sixteen cases of fresh single in-

TABLE II  
AUTOPSIED CASES

Total	46
With EKG	31
Single fresh infarct—anterior	13
Single fresh infarct—posterior	3
Old and recent infarcts	8
Old without recent infarcts	6
Sclerosis—infarct—no occlusion	1

\*The autopsy protocols have been dictated by several pathologists over a number of years. Personal inclination and interest have caused a variable degree of thoroughness in describing the pathologic changes. The usual autopsy routine may well fail to reveal all areas of previous infarction or occlusion of minor coronary branches. This objectionable feature has also been commented upon by Sprague and Orgain.

infarction were studied in detail. Thirteen were of the anterior type at autopsy and 3 of the posterior type. We have attempted to correlate the electrocardiograms obtained in these cases with the actual location of the infarction. Using this method, a majority of the curves were found to be of surprisingly little help, or of questionable help in the actual localization of the infarction as demonstrated at autopsy (Table III).

TABLE III  
EKG LOCALIZATION OF 16 CASES OF FRESH SINGLE INFARCTION  
Standard 3 Leads Only

EKG	ANTERIOR INFARCTION	POSTERIOR INFARCTION
Consistent	3	2
Doubtful	8	
Inconsistent	2	1

Table IV summarizes the 16 cases of fresh single infarctions.

It is remarkable to note that patients showing marked myocardial destruction, leading in some cases to rupture, could show only minor changes in electrocardiograms obtained shortly before death. Thus, in Case 3, the electrocardiogram of May 22, 1925, was normal, but the patient died on May 30, 1925, of a ruptured myocardium. Some of these curves studied critically with no knowledge of the actual location of the infarction, might be fairly stated to resemble the  $Q_1T_1$ , or posterior type of infarction, while the autopsy disclosed anterior infarction instead. It is appreciated that in many cases one electrocardiogram will not suffice and that progressive changes from day to day are frequently more significant. These records point to the need for caution in the interpretation of single electrocardiograms. No case showing typical  $Q_1T_1$  or anterior infarction change in the electrocardiogram was proved otherwise at autopsy.

Since 1935, we have been using the precordial leads as outlined by Wood and Wolferth and by Wilson. The technique of obtaining Lead IV has been standardized by an international committee,<sup>9</sup> and since January, 1938, the recommended method F4 has been followed in this clinic. However, in the material presented here Lead IV was obtained by a now discontinued method. That is, the right arm electrode was placed over the precordium. We were accustomed to locate this electrode at both the cardiac apex and in the left fourth interspace at the sternal border. The indifferent electrode was placed in the left interscapular region. Thus in these records a prominent  $Q_4$  and an inverted  $T_4$  are considered to be normal.

Of the 16 instances in which autopsy disclosed single fresh infarction, 5 were studied with the precordial lead (Lead IV) as well as with the standard leads. In 3 of these, Lead IV (Cases 9, 10, and 13) gave important evidence not available in the axial leads. In these 3 cases,  $Q_4$  was absent, and in 2 of them, in addition, the T wave was upright. In the remaining 2 records Lead IV was suggestive only. One of them showed depressed  $ST_4$ , though  $Q_4$  was present, while in the other,  $Q_4$  was likewise present but the T wave was upright.

Hence the information obtained with Lead IV was not misleading in any instance. On the contrary, in those cases where the axial leads were not in

TABLE IV

CASE NO.	PAIN	EKG	AUTOPSY
1	7/13/32	7/19/32 Small Q <sub>2</sub> and Q <sub>3</sub> Indeterminate	7/27/32 Infarction—left ventricle and septum Vessels—left descending occluded; right, marked sclerosis
2	11/14/28	11/16/28 S.T. interval depressed all leads Small Q <sub>2</sub> and Q <sub>3</sub> Indeterminate	12/ 1/28 Infarction—apex at left ventricle Vessels—ostia left not visible; right, pin point, caliber vessels open
3	5/ 8/25	5/22/25 Normal	5/30/25 Infarction—apex left ventricle with rupture Vessels—left descending occluded; right, no obstruction
4	3/24/30	4/ 5/30 S.T. interval depressed Lead I, II, elevated in III Prominent Q <sub>2</sub> Suggests posterior	6/ 6/30 Infarction—apex left ventricle and septum Vessels—left, descending occluded; right, no obstruction
5	6/16/28	6/18/28 S.T. interval depressed Lead I, elevated in III Prominent Q <sub>2</sub> Inconsistent	6/20/28 Infarction—anterior wall left ventricle Vessels—left circumflex occluded; right, no obstruction
6	3/22/29	4/ 9/28 T wave acutely inverted Lead I Small complexes Consistent	5/ 2/29 Infarction—anterior wall left ventricle Vessels—left descending occluded; right, sclerotic but patent
7	12/ 9/28	12/30/28 Small complexes Flat T-Waves Indeterminate	1/ 2/29 Infarction—anterior wall left ventricle Vessels—left occluded at bifurcation; right, no obstruction
8	None	3/29/28 Left axis deviation T waves inverted Lead I S.T. interval depressed Leads II, III Suggests anterior	4/ 3/28 Infarction—anterior wall left ventricle Vessels—left circumflex occluded; right, sclerotic but patent
9	10/20/35	11/29/35 QRS complex, relatively small and slurred Lead IV, absent Q, upright T Axial indeterminate Lead IV consistent	12/ 1/35 Infarction—anterior wall left ventricle with rupture Vessels—left descending occluded; right, sclerotic marked patent
10	6/12/36	6/26/36 Diphasic T-waves Leads II, III Lead IV, absent Q-wave Axial indeterminate Lead IV Consistent	7/ 2/36 Infarction—apex left ventricle Vessels—left descending occluded; right, sclerotic marked occluded
11	2/ 8/35	2/14/35 S.T. interval elevated Lead I, depressed Lead III Prominent Q <sub>1</sub> Diphasic T-wave Lead I Lead IV, S.T. interval depressed, small Q wave. Axial consistent Lead IV consistent	4/15/35 Infarction—anterior wall left ventricle Vessels—left occluded

TABLE IV—(CONT'D)

CASE NO	PAIN	ECG	AUTOPSY
12	12/24/34	12/27/34 ST interval elevated Lead I, depressed II III Shunting of S wave all leads Flat T wave Lead I Lead IV, absent Q in right T Axial consistent Lead IV consistent	1/18/35 Infarction—anterior wall left ventricle and septum Vessels—left descending occluded right, sclerotic but patent
13	5/23/36	5/24/36 ST interval depressed Leads I II Prominent Q <sub>1</sub> Lead IV, upright T wave Axial suggests posterior Lead IV suggests anterior	5/27/36 Infarction—apex left ventricle Vessels—left occluded, right sclerotic but patent
14	None	5/10/38 QRS complex relatively small and slightly bizarre Prominent Q <sub>1</sub> and Q <sub>2</sub> Axial consistent	5/2/38 Infarction—wall right ventricle Vessels—left no striking changes right, occluded
15	10/23/31	11/7/31 ST interval depressed Lead I, elevated in II III Prominent Q and Q <sub>2</sub> Acutely inverted T and T <sub>2</sub> Axial consistent	11/9/31 Infarction—left ventricle Vessels—left few plaques patent, right, occluded
16	None	12/10/30 Auricular flutter Indeterminate	1-12/30 Infarction—wall right ventricle Vessels—right occluded

diagnostic of the location or even of the presence of myocardial infarction Lead IV was of distinct help. Even with the additional precordial lead, the occasional case will not be entirely clear.

#### SUMMARY

This paper attempts to correlate present knowledge concerning electrocardiography with the diagnosis, prognosis and localization of myocardial infarction. Several factors are worthy of mention. In the diagnosis of proved myocardial infarction the standard three lead electrocardiograms may accurately localize the infarction, may suggest infarction without accurate localization, may falsely suggest the area involved, or may show no change whatever. The addition of progress readings and of precordial leads will clarify many of these cases. In this series where the information is based chiefly on the axial leads only, posterior infarction carries practically as high an immediate and subsequent mortality as does the anterior. The careful work of Saphir and his associates points to the need for more careful and detailed analysis of both the myocardium and the coronary atherosclerosis at autopsy. Wider recognition of this need may well explain some of the inconsistencies between the electrocardiograms and the autopsy protocols indicated in this paper.

#### CONCLUSIONS

1. A review of 83 expired cases of coronary artery thrombosis which were studied electrocardiographically does not indicate any material prognostic



difference between  $Q_1T_1$ , or anterior type of electrocardiogram and the  $Q_3T_3$  or posterior type.

2. The use of the standard three leads in coronary artery thrombosis without the addition of precordial leads has proved unsatisfactory in both the diagnosis and localization of myocardial infarction.

3. The addition of precordial leads gave definite indication of anterior myocardial infarction when the standard three leads alone did not, and in the cases where precordial leads were used there were no contradictory findings at autopsy.

#### REFERENCES

1. Wilson, F. N., MacLeod, A. G., Barker, P. S., Johnston, F. D., and Klostermeyer, L. L.: The Electrocardiogram in Myocardial Infection With Particular Reference to the Initial Deflections of the Ventricular Complex, *Heart* 16: 155, 1933.
2. Levine, S. A.: Coronary Thrombosis: Its Various Clinical Features, *Medicine* 8: 245, 1929.
3. Diseases of the Coronary Arteries and Cardiac Pain, Edited by Robert Levy, New York, 1936, Macmillan Co.
4. Barnes, A. R., and Whitten, M. B.: Study of the R-T Interval in Myocardial Infarction, *Am. Heart J.* 5: 142, 1929.
5. Parkinson, J., and Bedford, D. E.: Successive Changes in the Electrocardiogram After Cardiac Infarction (Coronary Thrombosis), *Heart* 14: 195, 1928.
6. Wood, F. C., Bellet, S., McMillan, T. M., and Wolferth, C. C.: Electrocardiograph Study of Coronary Occlusion, *Arch. Int. Med.* 52: 752, 1933.
7. Sprague, H. B., and Orgain, G. S.: Electrocardiographic Study of Cases of Coronary Occlusion Proved at Autopsy at the Massachusetts General Hospital, 1914-1934, *New England J. Med.* 212: 903, 1935.
8. Saphir, O., Priest, W. S., Hornburger, W. W., and Katz, L. N.: Coronary Arteriosclerosis, Coronary Thrombosis, and the Resulting Myocardial Changes, *Am. Heart J.* 10: 567, 762, 1935.
9. Standardization of Precordial Leads, *Miscellany, J. A. M. A.* 110: 395, 1938.

## THE CHEMISTRY OF THE URINE AFTER RENAL DECAPSULATION FOR ANURIA\*

M. MUSCHAT, D. MERANZE, AND H. ABRAMS, B.A., M.D., PHILADELPHIA, PA.

WE OBSERVED a case of anuria (five days of oliguria and two days of complete anuria) with complete recovery after decapsulation of one kidney. During this period the other kidney remained anuric, its function returning two weeks later. The entire output of urine was collected via the nephrostomy tube for a period of ten days. Further exact collections of urine were impossible because of removal of the nephrostomy tube. It was our intention to study the chemical composition of the urine over the entire ten-day period in order to ascertain the rapidity of excretion of the various chemical components after renal inactivity, and thus to obtain a picture of the degree of renal recovery.

T. F., No. A 1561, white female, aged 40 years, was admitted to the Mount Sinai Hospital on Dec. 2, 1937, and discharged Jan. 9, 1938.

\*From the Urological Service of the Mount Sinai Hospital, the Medical School of the University of Pennsylvania and the Department of Laboratories, Mount Sinai Hospital, Philadelphia.

Received for publication, April 21, 1938.

The patient was admitted for operative cystoscopy. Intravenous urography revealed a small calculus in the left ureter, about 5 cm above the vesical orifice. Both kidneys excreted the dye in normal concentration. The left urinary tract was notably dilated above the stone.

At cystoscopy under nitrous oxide anesthesia the tight orifice of the left ureter was opened with electrical knife. Some bleeding was noted, but not more than usual. The patient left the operating room in good condition. The patient was voiding 2 to 3 ounces of slightly bloody urine every half hour. About four hours later the patient had a vomiting spell and became pulseless. No blood pressure could be obtained. Intravenous fluids were administered,

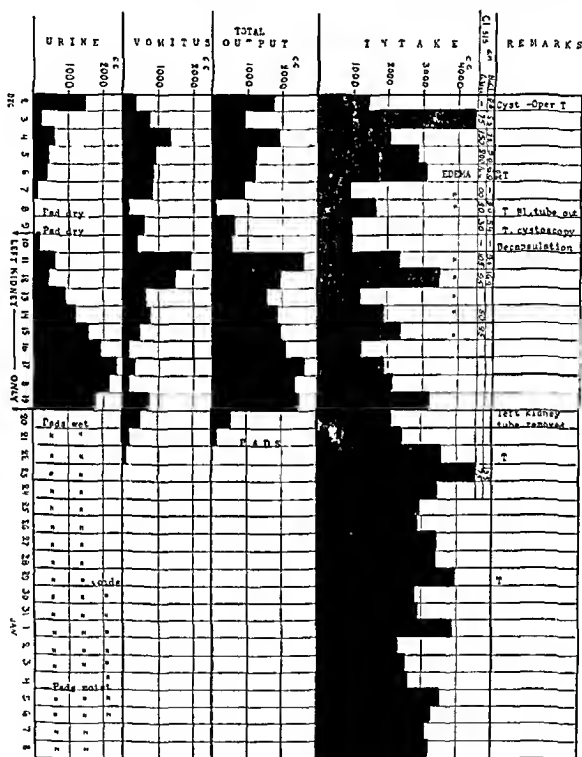


Fig 1—Master chart showing intake, total output vomitus and urine, and the total intake of sugar and chlorides

and the blood pressure returned to normal. On examination the bladder was found tremendously distended. Through a cystotomy incision about a quart of coagulated blood was evacuated from the bladder, the bleeding was coming from a small lively spurting artery at the mouth of the left ureter. Hemorrhage was stopped by suturing, and the wound was closed around a mushroom tube.

In spite of parenteral fluid administration vomiting persisted and the output continued very low (see Fig 1) for the next five days, and finally complete anuria for two days. Left

renal decapsulation was performed on Dec. 10, 1937. The kidney was much enlarged and very hard. The kidney tissue bulged out markedly when the capsule was opened. The tissue was pale brown, evidence of low blood circulation. Following this procedure, the urine began flowing, mounting rapidly with the gradual complete disappearance of all symptoms.

## COMMENT

These studies show some interesting facts (Fig. 2). The urinary output is gradual but slow in mounting in spite of the excess of fluids in the body. The specific gravity shows concentration with gradual dilution, the pH being slightly alkaline for the first two days, down to 5.6 during the next two days, back to

TABLE I  
CHEMISTRY OF THE BLOOD BEFORE AND AFTER DECAPSULATION

	BEFORE	AFTER		
	12/9	12/11	12/13	12/22
B.U.N. (mg.)	100		62	115
Creatinine (mg.)	7.8			6.8
Uric acid (mg.)	8.0			
Carbon dioxide (vol. per cent)	56			49
Sugar (mg.)	185			
Chlorine (mg.)	420	380	385	550
Calcium (mg.)	8.2			
Total protein (gm.)	5.6			
Serum albumin (gm.)	4.3			
Serum globulin (gm.)	1.3			

neutrality and low alkalinity where it stayed until the ninth day, when it dropped back to the normal acidity. There was no sugar in any of the specimens in spite of a great excess of sugar in the tissues. Urea showed the usual slow excretion and was still low on the tenth day. The blood urea nitrogen was still 115 on the tenth day. Creatinine was excreted slowly, but much more

TABLE II

GROUPING OF URINARY COMPONENTS INTO THRESHOLD CLASSES AND PERCENTAGE OF THEIR EXCRETION AS COMPARED WITH NORMAL

EXCRETION IN 24 HOURS					
		NORMAL	MAXIMUM	PER CENT	UNIT
High threshold	Glucose	0	0	0	
	Calcium	200	85.3	42.6	mg.
	Chloride	12	3.4	28.1	gm.
	Sodium	2.9	2.3	100.0	gm.
Low threshold	Urea	30.0	10.97	36.0	gm.
	Uric acid	700.0	726.8	100.0	mg.
	Phosphorus	2.5	3.34	100.0	gm.
No threshold	Creatinine	1200.0	1097.5	100.0	mg.
	Sulfur	3.0	3.3	100.0	gm.

rapidly than the urea, and reached normal on the tenth day. Uric acid behaved in the same manner as creatinine. Phosphorus, calcium, and chloride excretion were parallel and gradually mounted.

The loss of chlorides by constant vomiting before operation and for six days postoperatively was apparently not adequately balanced in spite of the saline elyses and hypertonic sodium chloride.

The present conception of the formation of urine is that the urine results from the glomerular filtration into Bowman's capsule of all the elements of the blood serum, except its protein content, and from the selective reabsorption of water and certain of the filtered substances by the tubules (Richards). Rehberg divides the soluble urinary constituents into three classes:

(1) Substances actively reabsorbed. These are conserved by the body and are present in lower concentration in the urine and in higher concentration in

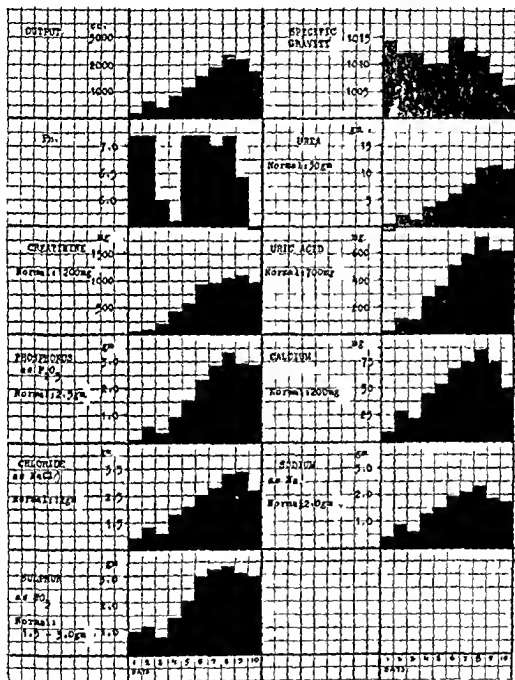


Fig. 2.—The excretion of the urinary components per twenty-four hours during the ten-day period

the reabsorbed fluid than in the plasma. These are the so-called "high threshold substances," among which are included glucose, calcium, chloride, and sodium.

(2) Substances which diffuse through the tubular epithelium when the concentration in the tubular fluid is greater than in plasma. These are never actively reabsorbed. They are never found in lower concentration in the urine or in higher concentration in the reabsorbed fluid than in plasma. These are the so-called "low threshold substances," among which are urea, uric acid, and phosphates.

(3) Substances which are neither reabsorbed nor back diffused, the so-called "non-threshold substances," like creatinine and sulfates.

We have included in our analysis of the urine collected in the postdecapsulation period, representatives from these three classes of threshold substances. In general, it appears that at the end of the ten-day period, at which our study in its planned form had to be discontinued, the kidney had essentially recovered its functional integrity. At this period the kidney was excreting normal average quantities of at least one representative of each of the three classes listed above. The exceptions, calcium, chlorides, and urea, are not difficult to explain. The low calcium and chloride quantities may represent successful conservation of these substances by the body, since at this time the intake of these elements was below average in quantity. The patient was still losing chlorides by vomiting. The low urea output is not difficult to explain. The urinary output was still too low to have washed out the entire amount of urea accumulated in the blood since urea requires large amounts of water for complete elimination.

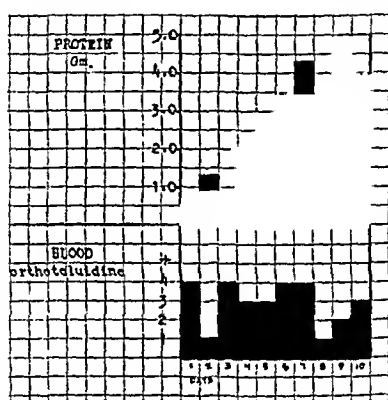


Fig. 3.—Parallel determination of blood and protein in the urine during the ten-day period to ascertain their quantitative relationship. It shows the amount of protein to be greater and out of proportion with the amount of blood, indicating true and heavy albuminuria.

It would appear that tubular function recovered very quickly since even on the first day no glucose appeared in the urine, and that the output was limited and conditioned chiefly by glomerular function, the latter limited, perhaps, in activity by the gradual reestablishment of adequate glomerular circulation. Thus, even at the end of the ten-day period, uncorrelated blood cells and albumin were present in the urine (Fig. 3).

Much of our findings can be adequately explained on the supposition that glomerular function was only gradually and imperfectly restored, while tubular function was quickly and satisfactorily regained.

There is no question that the decapsulation and that this procedure alone has restored the flow of urine from the kidney since the other kidney, remaining untouched, did not begin functioning spontaneously until two weeks later.

A pertinent question presents itself. Did the inactivity during the anuric state cause any alteration in either the glomerular or tubular function, or was the kidney stopped in its function just like stopping a clock and restarted without ill effect? Glomerular alteration should have evidenced itself by the presence

of albumin in the urine after decapsulation. Albumin was found in every specimen. Tubular injury would have evidenced itself by the presence of sugar in the urine. We found no evidence of sugar postoperatively.

It is apparent, therefore, that only one vital mechanism of the kidney was injured, namely, the glomerulus, while the tubular function was not altered.

One is unable to explain the cause of the anuria in this instance. We can only surmise that the glomerular function ceased because of tremendous swelling of the kidney, gradually increasing the tension within the renal capsule until the blood flow to the glomerulus ceased. This is supported by the observation on the operating table when we found the kidney pale brown and not red, evidence of great reduction in the quantity of circulating blood.

The blood pressure was either not high enough to overcome the greatly increased intrarenal pressure on the other side of the glomerular filter, or if it was sufficient and did permit normal filtration to proceed, the filtrate could not enter the compressed tubuli and further filtration ceased.

From all the data obtained, we must conclude that the anuria in this case was due to an idiopathic intrarenal edema. The edema was gradual in growth, as seen from the gradual decline of the urinary output, until the pressure became so great that by complete compression of the afferent blood supply to the glomeruli total anuria ensued. In order to determine the *modus operandi* of the vascular mechanism in anuria, several questions must be answered in the individual case of anuria: (1) Does the renal artery pulsate? (2) Does the renal cortex bleed? (3) Is there blood leaving the renal veins?

We are unable to answer these questions in this particular case as we did not think of them during the rapid operative procedure of the decapsulation. But these questions should be in the mind of every surgeon who is confronted with a case of anuria. Only by answering them correctly in many instances will the problem of true renal anuria be solved. The palpation of the renal artery is simple and quick in execution. The bleeding test of the cortex should not consume more than a few seconds. By making a few small superficial stab incisions, one can convince himself whether the cortex is rich or poor in blood. The observation on the renal vein is more difficult. One has to visualize the vein and by milking it, determine whether or not there exists an active return blood flow from the kidney.

By accumulating such data, the problem of true renal anuria will narrow itself down to a few causative agents and will thus enhance its future prevention and therapy.

# CLINICAL OBSERVATIONS ON THE VALUE OF VARIOUS XANTHINE DERIVATIVES IN ANGINA PECTORIS\*

H. M. MASSEL, M.D., CHICAGO, ILL.

ASKANAZY<sup>1</sup> in 1895 was the first to report beneficial results with xanthines in angina pectoris. Since then a number of similar reports have appeared.<sup>2-7</sup> Evans and Hoyle<sup>8</sup> found that the xanthines did not compare in efficiency with glyceryl trinitrate in patients with angina pectoris. Riseman and Brown<sup>7</sup> concluded that the patients' reaction to various xanthines was too unreliable to judge their efficacy and to distinguish their comparative value. Placebos were found to give about the same degree of subjective relief as the various xanthines. They proposed to check the benefits objectively, using an exercise tolerance test leading to anginal pain as a measure of the results. With this objective method they reported benefits with xanthines in some patients, but the results with these drugs were not comparable to glyceryl trinitrate. Unfortunately, in our hands, the exercise tolerance test has not worked satisfactorily in that we<sup>9</sup> have not obtained the frequency of positive tests in patients with angina pectoris reported by Riseman and Brown.<sup>7</sup> We have therefore resorted to the patients' comparative subjective reactions to the various xanthines, placebos, and barbiturates when several of these were used in the same patients in succession.

## PLAN OF STUDY

Fourteen patients were selected who were known to have angina pectoris, based on organic heart disease, for at least a year before starting the therapy. They were regular visitors in the Adult Cardiac Clinic in the Mandel Clinic and were able and willing to cooperate in this study by coming regularly and taking medication as prescribed. This study was carried on over a period of sixteen months beginning in June, 1936. Each of the 14 patients was placed on from three to fourteen courses of treatment, each course consisting of three weeks' medication of a single drug or a combination of drugs. The medication was taken in tablets four times daily, the tablets being given the patient directly by the investigator in such a way that the patient did not know the nature of the drugs given. No attempt was made to alter the handling of the patient in any other regard.

## RESULTS

Table I summarizes the various courses of medication in the different patients classified according to the patient's report of the effect of the drug. It will be seen that they fall into four courses: xanthines (A, B, C), pheno-

\*From the Cardiovascular Department, Michael Reese Hospital, Chicago. Aided by the A. D. Nast Fund for Cardiac Research.  
Received for publication, May 16, 1938.

barbital (F, H), placebos (D, E), and combinations of xanthines with phenobarbital (I, J, K, L)

TABLE I  
EFFECT OF DRUGS ON ANGINA PECTORIS

PATIENT	SATISFACTORY RELIEF OBTAINED WITH THE FOLLOWING MEDICATION	MILD RELIEF OBTAINED WITH THE FOLLOWING MEDICATION	NO RELIEF OBTAINED WITH THE FOLLOWING MEDICATION
1	A, A, L, L	B	B, E, K
2	B, F		A, B, B, C, D, D, E
3	A		C, E, F, L
4	C, I, I, I, I, J, K, K, L	A	B, C, C, H
5	A, A, A		E
6	A, B, C, J, K	B, D, D, K, L	B, E, F, I
7	C, C, D, E		B, B, D, F
8	I, I, I		K
9	A		A, A
10	J, L		H
11	I, I, I, K, L		H
12	F		J, K, L
13	H, I, I, K		A, E, F, F, H, I
14	I, I, I		F

A Theocalcin theobromine calcium salicylate gr vi ss qid Billhuber Knoll & Co  
 B Phyllin (theophyllin calcium salicylate) gr v qid Billhuber Knoll & Co  
 C Aminophyllin (theophyllin ethylenediamine) gr i ss qid Dubin Laboratories  
 D Acetyl salicylic acid gr v qid  
 E Sodium bicarbonate gr x qid  
 F Phenobarbital gr ss qid  
 H E plus F  
 I Theobromine sodium salicylate gr v with phenobarbital gr ss qid  
 J C plus F  
 K B plus F  
 L A plus F

Table II shows the results with the xanthines, Table III the results obtained with placebos and phenobarbital, and Table IV the results obtained with combinations of xanthine and phenobarbital

TABLE II  
EFFECT OF XANTHINES ALONE ON ANGINA PECTORIS

DRUG COMBINATION	PATIENTS	COURSES	COURSES GIVING RELIEF	COURSES GIVING NO RELIEF
A*	8	13	9 (69 per cent)	4 (31 per cent)
B*	5	12	5 (42 per cent)	7 (58 per cent)
C*	5	8	4 (50 per cent)	4 (50 per cent)
Total		33	18 (55 per cent)	15 (45 per cent)

\*See Table I footnote

TABLE III  
EFFECT OF PLACEBOS AND PHENOBARBITAL ON ANGINA PECTORIS

DRUG COMBINATION	PATIENTS	COURSES	COURSES GIVING RELIEF	COURSES GIVING NO RELIEF
D*	3	5	2 (40 per cent)	3 (60 per cent)
E*	7	8	1 (13 per cent)	7 (87 per cent)
F*	6	7	2 (29 per cent)	5 (71 per cent)
H*	4	5	1 (20 per cent)	4 (80 per cent)
Total		25	6 (24 per cent)	19 (76 per cent)

\*See Table I footnote



TABLE IV

EFFECT OF COMBINATION OF XANTHINES WITH PHENOBARBITAL ON ANGINA PECTORIS

DRUG COMBINATION	PATIENTS	COURSES	COURSES GIVING RELIEF	COURSES GIVING NO RELIEF
I*	6	17	15 (88 per cent)	2 (12 per cent)
J*	4	4	3 (75 per cent)	1 (25 per cent)
K*	7	9	6 (67 per cent)	3 (33 per cent)
L	7	8	6 (75 per cent)	2 (25 per cent)
Total		38	30 (79 per cent)	8 (21 per cent)

\*See Table 1 footnote.

The following were the criteria used in establishing the benefits of medication: Satisfactory relief with one or more drugs was considered present when the patient reported that he was able to do his usual day's work with little or no precordial pain, and felt that the medicine he was taking was giving him satisfactory relief, so that he used no nitroglycerin except on rare occasions. Mild relief with the medication was considered present when the patient volunteered the information that he received relief at times and had reduced his nitroglycerin medication considerably. No relief with a drug combination was considered present when the patient noted very little or no relief and reported the need of nitroglycerin in the usual amounts.

On this basis it was found that phenobarbital, sodium bicarbonate, and their combination, as well as aspirin, gave relief in about one-fourth of the courses. The xanthines gave relief in one-half the courses, while the combination of xanthine with phenobarbital gave relief in four-fifths of the courses. Theocalcin alone is an exception to the other xanthine derivatives used in that relief was obtained in seven-tenths of the courses.

## SUMMARY

These results are significant in indicating that:

(1) The xanthines used, especially theocalcin, offer a better chance for relief of angina pectoris, in the ratio of 2 to 1, than aspirin, sodium bicarbonate, phenobarbital, or a combination of the latter two.

(2) The combination of a xanthine with phenobarbital offers a much better chance for relief of angina pectoris, in the ratio of more than 3 to 1, than the phenobarbital alone or in combination with sodium bicarbonate.

(3) The combination of a xanthine with phenobarbital offers a better chance for relief of angina, in the ratio 1.5 to 1, than xanthine alone.

(4) While the errors inherent in such types of studies as these are large and the series of courses of therapy used are relatively small, the results, we believe, cannot be dismissed as without significance.

(5) It is, therefore, concluded that xanthine derivatives do have a beneficial effect on the subjective state of the patient with angina pectoris and that this effect is definitely enhanced when the xanthine is combined with a sedative like phenobarbital.

425 S. PULASKI RD.

I wish to acknowledge my indebtedness to Dr. Louis N. Katz at whose suggestion this study was undertaken, for his guidance in the conduct of the investigation and for his advice in preparing this report. I am indebted to Dr. W. C. Buchbinder for his advice in the selection of patients for this study.

The Bilhuber-Knoll and Co., and the H. E. Dubin Laboratories, Inc., supplied some of the xanthine derivatives for this study.

## REFERENCES

- 1 Aschanzy, S. Klinisches uiber Diuretin. Deutsches Arch f Klin Med 56 209 1899
- 2 Musser, J. H. Theophylline Ethylenediamine in Heart Disease Associated With Pain. J A M A 91 1242, 1928
- 3 Gilbert, N. C. and Kerr, J. A. Clinical Results in Treatment of Angina Pectoris With Purine Base Diuretics, J A M A 92 201 1929
- 4 Coogan, T. J. Some Clinical Observations on the Uses of Theophylline Calcium Salicylate, Tr Am Therap Soc 34 137 1934
- 5 Smith, F. M., Rathe, H. W., and Paul, M. D. Theophylline in Treatment of Disease of Coronary Arteries, Arch Int Med 56 150 1935
- 6 Gilbert, N. C. Treatment of Angina Pectoris. M Clin North America 19 1085 1936
- 7 Riseman, J. E. F., and Brown, M. G. Medicinal Treatment of Angina Pectoris, Arch Int Med 60 100, 1937
- 8 Evans, W., and Hoyle, C. Prevention and Treatment of Individual Attacks of Angina Pectoris (Angina of Effort), Quart J Med 3 105 1934
- 9 Robertson, S., and Kritz, L. N. Observations on Referred Pain of Cardiac Origin, Am J M Sc 196 199, 1938

## THE EFFECTS OF BACTERIAL TOXINS ON ERYTHROGENESIS\*

## A STUDY OF THE PERIPHERAL BLOOD PICTURE IN RABBITS MADE ANEMIC BY CHRONIC HEMORRHAGE

GEORGE W. WILLISON, M.D. BOSTON, MASS

IN THE normal individual a remarkably constant balance between the forces of blood destruction and blood regeneration is maintained. This physiologic equilibrium is subject to the influence of every disease process in the body, local or systemic, by reason of the special functions of the blood and its distribution to all parts of the body. Of the significant disturbances the most important general group is that of the anemias. On the basis of recent advances in hematology, and by the correlation of clinical and laboratory studies, Haden<sup>1</sup> has suggested the following classification of the anemias: (1) anemia due to acute hemorrhage, (2) anemia caused by excessive blood destruction, (3) anemia resulting from depression of bone marrow function, and (4) anemia due to the absence of specific substances necessary in the building of normal erythrocytes. Often a combination of these factors is at work in the production of an anemia. In the first group mentioned the cause of the anemia is obvious. The fourth general group includes those anemias which result from specific deficiencies in the building materials and catalyst like substances which are necessary in the formation and release of normal erythrocytes to the peripheral circulation. The results of deficiencies in two such elements, namely, iron and antianemic substance, are definitely recognized. Sabin<sup>2</sup> has shown the points of action of these two substances in the maturation of the normal red blood cell. The great majority of the anemias, however, are included in groups 2 and 3. Anemias resulting from excessive erythrocyte destruction within the body may

\*From the Lilly Laboratory for Clinical Research in Indianapolis City Hospital and the Department of Medicine, Indiana University School of Medicine, Indianapolis.

This study was made possible by a grant provided by the Henry Strong Denison Foundation for Medical Research.

Received for publication May 23 1938

be due to hemolytic poisons such as mushrooms, nitrobenzol, and snake venom, to bacterial toxins, to destruction of red cells by malarial and other parasites, or to abnormal erythrocyte fragility. Marrow poisons, such as benzol and lead, bacterial toxins, excessive irradiation of the bone marrow, and invasion and replacement or destruction of marrow by other tissue (osteosclerosis, malignancy, leucemia, etc.) are recognized as causes for the anemias due to depressed bone marrow activity.

It is significant that bacterial toxins appear as causative factors in both groups 2 and 3. Infections are unquestionably the greatest single cause of anemia and they produce the greatest variety of clinical types. So variable are the results of clinical and laboratory studies within this group that they possess no group diagnostic value. The mechanism by which anemic states are produced by bacteria and their toxins has had no adequate explanation. Present evidence would seem to indicate that there are probably a concomitant depression of the bone marrow and an increased red cell destruction.<sup>3</sup>

Further studies on the etiologic relationship of bacterial toxins to the anemias would be valuable. The present investigation was made with this thought in mind. In this series of experiments the data have been obtained by study of the peripheral blood picture. Investigations by Steele<sup>4</sup> and others<sup>5,6</sup> have shown the close correlation of the bone marrow pattern and the peripheral blood picture. The reticulocyte count is a sensitive and reliable index to the state of erythrocytic activity of the bone marrow. Steele said, "The marrow finds direct expression in terms of the cells of the blood." For this investigation standard tetanus and diphtheria toxins, streptococcus and staphylococcus hemotoxins, and diphtheria toxoid were used.

#### METHOD

These studies were made on adult rabbits taken from stock. Any rabbit showing signs of disease, injury, or other abnormal change was discarded and its records were excluded from the general data. An adequate diet was given to the rabbits and water was available at all times.

Each rabbit was observed for a varying period to determine normal blood values. Red and white blood cell counts, hemoglobin values, reticulocyte counts, and rectal temperatures were taken at frequent intervals. The normal control period was followed by a period during which the rabbits were subjected to frequent bleedings, so regulated as to produce a chronic anemia with as constant a reticulocytosis as possible.

The rabbits were made anemic by repeated bleedings to stimulate reticulocyte production. In the presence of increased erythrocytic activity, variations in the degree of reticulocyte response are shown more strikingly and minor changes are more clearly discernible. Also, chronic hemorrhage creates a constant demand for red blood cells in the peripheral circulation. Erythrocytes are not stored, but are rapidly delivered to the peripheral blood, and thus a possible source of error is eliminated.

While in this chronic anemic state, the rabbits were given the various bacterial toxins. Subsequent red and white blood cell and reticulocyte counts and hemoglobin values were taken until the animal died or until significant blood changes had ceased. Concomitant records of weights and temperatures were kept.

Blood was drawn by puncture of a vein at the base of the ear. Standard pipettes and counting chambers were used in making the red and white cell counts. Hemoglobin levels were determined by the Newcomer method, on the instrument used, 100 per cent was equivalent to 15.6 gm of hemoglobin in each 100 cc of blood. Reticulocyte counts were made from blood smears prepared on cover slips stained with brilliant cresyl blue and counterstained with Wright's stain. These smears were also examined for variations in size and shape of the red blood cells and for the presence of normoblasts.

In administering the various toxins and bacterial products to the rabbits no standard procedure was followed. With the exception of the *Staphylococcus aureus* hemotoxin all injections were given subcutaneously, this product was injected into the muscles of the thigh. The dosages of the toxins used were varied. Doses of tetanus toxin, ranging from sublethal amounts up to several times the lethal dose, were given. By a process of trial and error the lethal dose of diphtheria toxin was determined and sublethal and finally lethal doses were given. Increasingly large doses of streptococcus and staphylococcus hemotoxins and of diphtheria toxoid were administered.

Necropsy was performed on all animals that died. Portions of tibial bone marrow, spleen, liver, and kidney were fixed in Zenker's and Carnot's solutions. The tissue was sectioned and stained with hematoxylin eosin and Giemsa stains.

#### THE EFFECTS OF TETANUS TOXIN

The rabbit possesses considerable natural resistance to tetanus toxin. When this toxin is injected subcutaneously, the subsequent developments depend upon the rate of administration as well as the total amount of toxin given. A single lethal dose will cause death in seven or eight days in the average rabbit. Five or six days after the injection of the toxin, muscle spasms develop near the site of the injection and gradually spread to the rest of the body. The muscle hypertonicity increases and convulsions occur after stimulation, followed by intervals during which the animal lies exhausted. Finally the animal dies in a state of extreme general tonic contraction. If the first or first few injections are not sufficient to overcome the rabbit's natural resistance and its rapidly developing acquired immunity, it does not succumb. When sublethal doses are employed in the beginning, the amount of toxin can be rapidly increased to many times the lethal dose without causing permanent damage to the rabbit.

The peripheral blood values of six rabbits were determined by frequent examinations for a period of several weeks and the animals then subjected to repeated bleedings. After the bleedings were begun, there was an immediate reticulocyte response in each case. The rapidity with which the reticulocyte percentage rose depended upon the degree of hemorrhage to which the animals were subjected and to individual differences in response. In general, the more quickly blood was taken, the quicker the response. For a few days the absolute number of reticulocytes per cubic millimeter was usually relatively less than the reticulocyte percentage because of the loss of many red blood cells. Early observations indicated that if the frequency and size of the bleedings were so regulated as to hold the red blood cell count between 3,500,000 and 4,000,000 per cmm, there would be established a fairly constant reticulocytosis, averaging about 20 per cent and equivalent to an absolute number of reticulocytes ranging

from 600,000 to 800,000 per c.mm. This anemic state could be maintained for months and reticulocytosis held at a high level. The anemic control period was considered that period between the first high point in the reticulocyte level and the beginning of toxin administration. Standard tetanus toxin was administered subcutaneously to the animals in varying doses and at various intervals. Blood studies were continued until the animals died or until significant changes in the peripheral picture had ceased.

The most striking observation was the marked contrast in the values obtained from those animals which had received a lethal dose of the toxin and those which had not. In the former group, during the period of intoxication a very dramatic drop occurred in reticulocyte production, whereas in the latter group this was slight and transient.

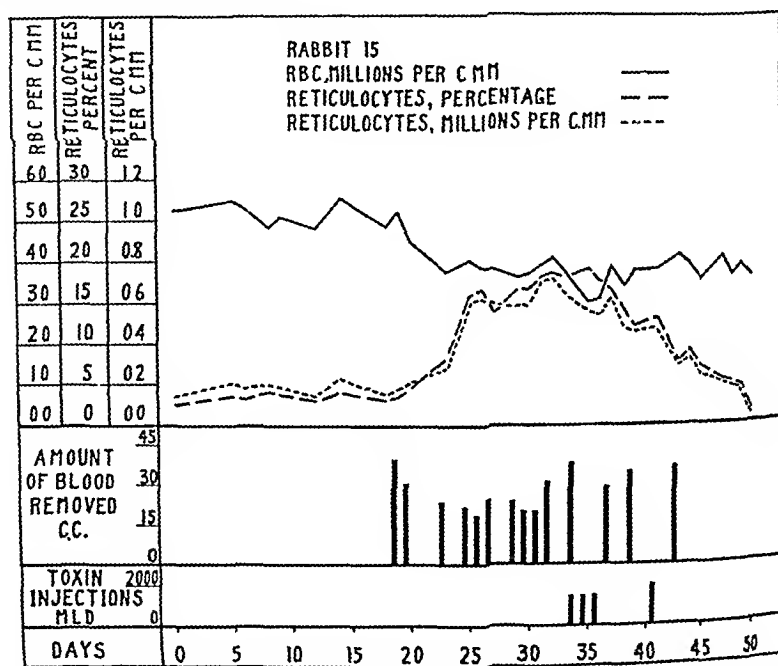


Chart 1—Observations on the blood of Rabbit 15 injected with tetanus toxin in lethal amount

During the anemic control period the average red blood cell count for animals receiving a lethal dose of the toxin was 1,760,000 per c.mm. lower than in the normal control period. The average reticulocyte count was 670,000 per c.mm., or almost five times greater than the normal value. The average daily bleeding during the anemic control period was 20.3 c.e. From the third to the seventh day following the administration of toxin a decline began in the reticulocyte percentage which was progressive until death occurred. The average red cell count of 3,410,000 per c.mm. at the time of the original injection of toxin corresponded closely to that at the termination of the period, 3,610,000 per c.mm., whereas the absolute number of reticulocytes had dropped off from an original 720,000 per c.mm. to a terminal 140,000, or more than a five-fold reduction. After the toxin injections were begun, rabbits receiving lethal amounts of the toxin were bled less often, and much less blood was removed than during

the anemic control period. During the period of intoxication the average daily bleeding was only 10.8 cc as compared to a daily average of 20.3 cc during the anemic control period. By so reducing the size and frequency of the bleedings following the toxin injections, the red cell count was maintained at the pret toxin level, but there was a marked, progressive decline in reticulocyte values. Chart 1 illustrates in graphic form the course of blood values in Rabbit 15, which is typical for the animals receiving a lethal dose of toxin.

The data for animals receiving sublethal doses of the toxin were very similar to those in the group above up to the point at which toxin administration was begun. Subsequently the two groups were in marked contrast. In the animals receiving sublethal intoxications only transient changes were noted in the peripheral

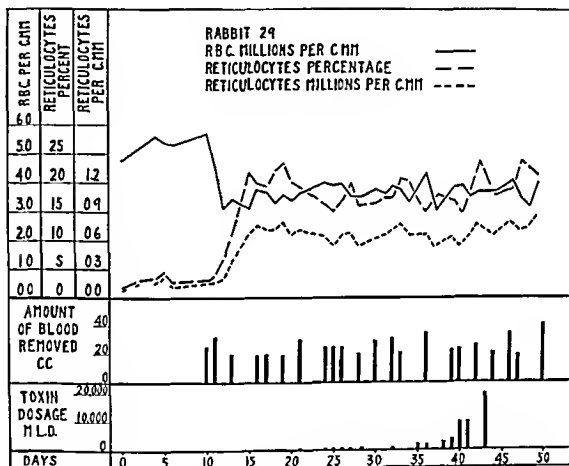


Chart 2—Observations on the blood of Rabbit 29 injected with tetanus toxin so as to produce sublethal intoxication

blood. After a short reticulocyte depression of varying degree reticulocytes again appeared in numbers equal to those present during the anemic control period. The average daily bleeding after toxin injection was 14.5 cc as compared to 16.8 cc for the anemic control period. Terminal red blood cell and hemoglobin values varied only slightly from pret toxin levels. In Chart 2 are presented blood values of Rabbit 29, which are typical for the animals receiving sublethal doses of the toxin.

In the early stage of the anemia in all rabbits numerous large reticulocytes, heavily laden with hemoglobin, were seen in the blood smears. After the anemia had existed for some time, occasional normoblasts were always present. Polychromatophilia and anisocytosis were constantly noted. The average final red cell count for all animals was 66 per cent of the control value, whereas the final hemoglobin level was 70 per cent of the normal value. Thus, in spite of long continued bleedings, the bone marrow delivered normal or slightly hyper

chronic red blood cells into the peripheral circulation. In some instances there was a slight temperature rise following the toxin injections, but these changes were not constant. When the intoxication was lethal, a subnormal temperature existed for some time before death. Without exception the animals gained in weight through the normal and anemic control periods. Those that died lost from 1½ to 3 pounds in the terminal stages. Those not receiving a lethal dose showed no characteristic or significant changes in weight. No constant variations in the white blood cell counts were noted.

At post-mortem examination the red marrow of the animals that died was definitely hyperplastic. Red marrow filled the cavity of the femur and occupied two-thirds or more of the proximal end of the tibia. Microscopic examination

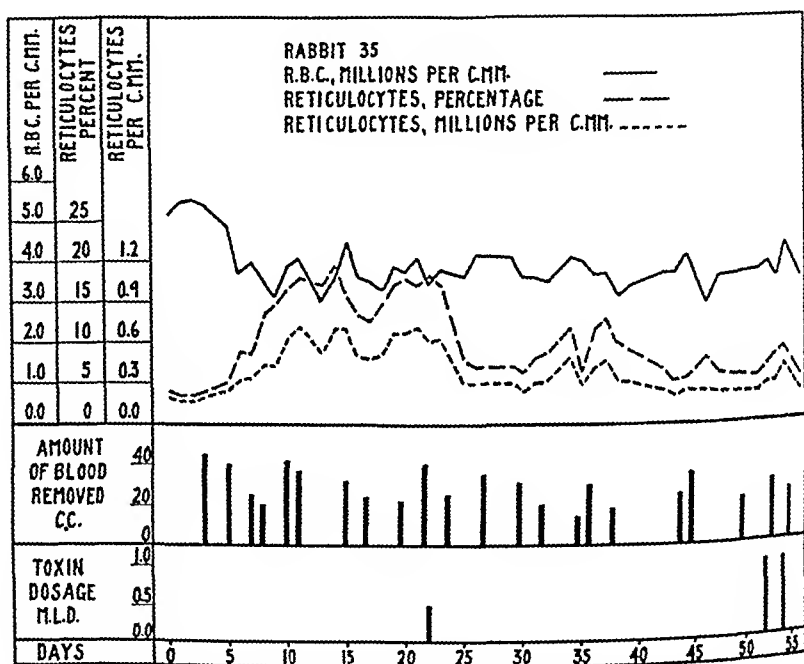


Chart 3.—Observations on the blood of Rabbit 35 injected with diphtheria toxin.

of certain sections of the bone marrow showed a highly cellular marrow and a decrease in fat content. The sinusoids were dilated and engorged, and there appeared to be an increase in the proportion of erythroid cells. There was relatively little nuclear division. Other marrow sections, probably from the lower third of the tibia, were not hyperplastic, indicating that there was still room for expansion of the hematopoietic function. Sections of the spleen did not show an unusual amount of phagocytosis of red blood cells or of nuclear debris. Some showed a moderate amount of material having the appearance of hemosiderin, but this was not particularly striking. Sections of the liver showed areas of toxic degeneration.

#### EFFECTS OF DIPHTHERIA TOXIN AND TOXOID

A lethal dose of diphtheria toxin injected subcutaneously into a rabbit will cause death in eighteen to ninety-six hours, depending upon the size of the

dose. If a series of smaller doses is given so as to produce lethal intoxication, the animal will survive longer and show progressive paralysis and cachexia.

The peripheral blood of three rabbits was examined frequently for a period of several days to determine normal blood values. A fairly constant anemia was established by chronic hemorrhage. Two of the animals were given subcutaneous injections of varying doses of diphtheria toxin, and the third received repeated doses of diphtheria toxoid.

At the beginning of the period of intoxication reticulocyte values for both rabbits receiving diphtheria toxin were high, namely an average of 690,000 per cmm, as compared to the normal value of 180,000. By means of several trials the lethal dose of toxin for a rabbit was determined. To one of the rabbits a lethal dose was administered in a period of four days. Immediately after the

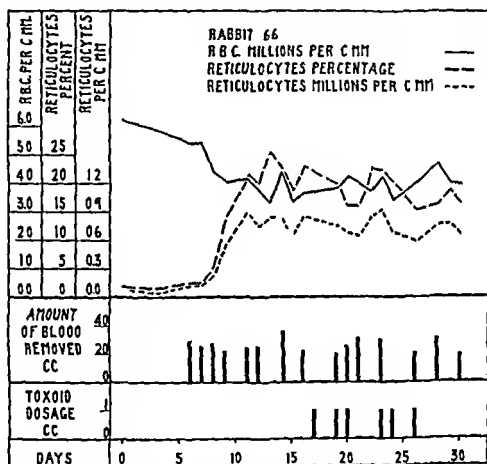


Chart 4—Observations on the blood of Rabbit 66 injected with diphtheria toxin 1

first injection there began a rapid reduction in the number of reticulocytes in the peripheral blood which continued until death. To the other rabbit a single sublethal dose was first given and the blood values followed for one month before a subsequent injection of toxin. During this period of observation and while the red blood cell level was maintained fairly constantly, there was a drastic reduction in the number of reticulocytes which persisted. Further fatal doses of the toxin produced a still greater drop of reticulocytes. Values for Rabbit 35 are shown graphically in Chart 3.

Although in the one case the period of intoxication was short and in the other it was prolonged, the blood values show very similar general trends. Whereas the average pretoxin red blood cell count was 3,310,000 and the absolute number of reticulocytes 690,000 per cmm, the terminal red blood cell count was 3,060,000 and the reticulocytes 210,000 per cmm. This more than threefold reduction in the reticulocytes would have been greater had the terminal red blood



cell count coincided more closely with the former, for, in these prolonged chronic anemias, additional bleeding with reduction of the red blood cell count always caused an increased outpouring of reticulocytes in the peripheral blood. Compared to an average daily bleeding of 14.6 c.c. during the anemic control period, only 9.5 c.c. were taken each day during the period of intoxication. Thus, while one-third less blood was being taken daily and the red cell count decreased slightly, a very conspicuous reduction occurred in the number of reticulocytes in the peripheral blood.

Examination of the blood smears revealed frequent normoblasts. In the later stages anisocytosis was prominent, and many of the cells were extremely hypochromic. The terminal hemoglobin value was 52 per cent of the normal value, and the red blood cell count was 57 per cent of the normal; hence, toward the end of the course there was a drop in the color index. During the period of intoxication there was a gradual weight loss, varying from  $1\frac{1}{4}$  to  $2\frac{3}{4}$  pounds. Each toxin injection was followed by a temporary temperature rise up to as much as  $1^{\circ}$  F. The toxin also produced a varying degree of leucocytosis. At necropsy an extension of the red marrow of these animals was apparent and the marrow showed definite hyperplastic changes.

Chart 4 shows the values in the rabbit given diphtheria toxoid. This material was administered subcutaneously in 1 c.c. doses and was repeated every second to fourth day until the animal succumbed. The variations in the red blood cell and reticulocyte counts were no greater than might have been expected had the diphtheria toxoid not been given. The percentage and absolute number of reticulocytes remained high until the death of the animal. Death was attended by an extreme diarrhea, with rapid dehydration and wasting. A striking fact in this case, although the bleedings were not unusually excessive or prolonged, was the low hemoglobin level. Whereas the final red blood cell count was 69.8 per cent of the normal control value, the terminal hemoglobin value was only 52.8 per cent of the original. Whether the diphtheria toxoid acted directly or indirectly to produce this marked lowering of the color index is a conjectural point.

#### EFFECT OF STREPTOCOCCUS AND STAPHYLOCOCCUS HEMOTOXINS

The pathogenicity of the soluble exotoxins of *Streptococcus pyogenes* for rabbits varies greatly, depending on the virulence of the strain. For this study the toxin of *Streptococcus scarlatinae* was chosen as fairly characteristic of the toxins produced by this group of organisms. The staphylococcus toxin was prepared from a strain of *Staphylococcus aureus* isolated from a furuncle. It was ascertained that this strain possessed a fairly high toxigenic capacity and produced a filtrable toxin which was hemolytic. The exact unit strength of these toxins was not determined, but they were considered to be representative of the two groups of organisms.

The peripheral blood values of four rabbits were determined by frequent examinations for several days, and the animals were then subjected to repeated bleedings. When the reticulocyte response had reached a high sustained level, two of the rabbits were given increasingly large doses of *Streptococcus scarlatinae* hemotoxin subcutaneously. The other two received increasing intramuscular injections of *Staphylococcus aureus* hemotoxin.

Chart 5 shows graphically the course of peripheral blood values of Rabbit 72, which is typical for both rabbits receiving streptococcus toxin. The total dosage given each of the animals was very large. Early small doses produced a reduction in the number of reticulocytes which was only temporary; to maintain the lower reticulocyte values the dosage had to be rapidly increased. This fact is best illustrated by the course of Rabbit 72 in which the reticulocytes after a transient depression rose almost to the pretoxin level and only after very large doses of the toxin did they drop to a low level. The average final value of reticulocytes in the peripheral blood of both animals was 380,000 per c.mm. as compared to pretoxin value of 870,000; this drastic reduction occurred with scarcely any change in the red blood cell count or in the hemoglobin percentage.

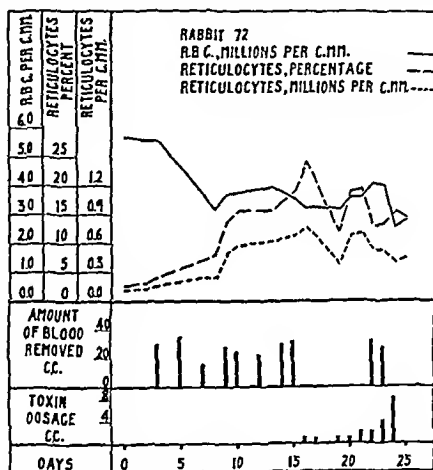


Chart 5.—Observations on the blood of Rabbit 72 injected with *Streptococcus scarlatinae* hemotoxin.

The average daily bleeding was only 7.6 c.c. during the period of intoxication as compared to 16.4 c.c. for the anemic control period.

The toxin injections occasionally caused a temporary rise in temperature (in one case, 2.5° F.), but this change was not constant. A small increase in the number of leucocytes followed the toxin administration. No significant weight changes were noted. Blood smears showed anisocytosis, polychromatophilia, and occasional normoblasts.

Chart 6 shows the course of blood values of Rabbit 68 which received staphylococcus toxin. For several days this animal was given relatively small doses of the toxin. After a rest period of ten days in which no toxin was given, injections of larger doses were resumed. The initial injections produced a decrease in the number of reticulocytes, but during the rest period it increased to the pretoxin level. When toxin administration was resumed, the reticulocytes again fell off rapidly. A second rabbit was given five increasingly large doses

of the toxin on consecutive days. In this case, there was a progressive reduction in the number of reticulocytes. The average reticulocyte count for both rabbits at the termination of the study was 360,000 as compared to 690,000 per c.mm. for the anemic control period and 530,000 just before the toxin administration. An average of 5.6 c.c. less blood was taken daily during the period of intoxication than during the anemic control period. The fact that as much blood was taken daily from Rabbit 68 after the beginning of toxin injections as before can be ascribed chiefly to the rest period of ten days between injections, when the reticulocytes reached the pretoxin level. Average values revealed that staphylococcus hemotoxin in large doses produced a definite reduction in the number of reticulocytes in the peripheral circulation.

Injections of this toxin called forth a moderate leucocytosis in both animals. Slight temperature elevations sometimes occurred following the toxin, but these

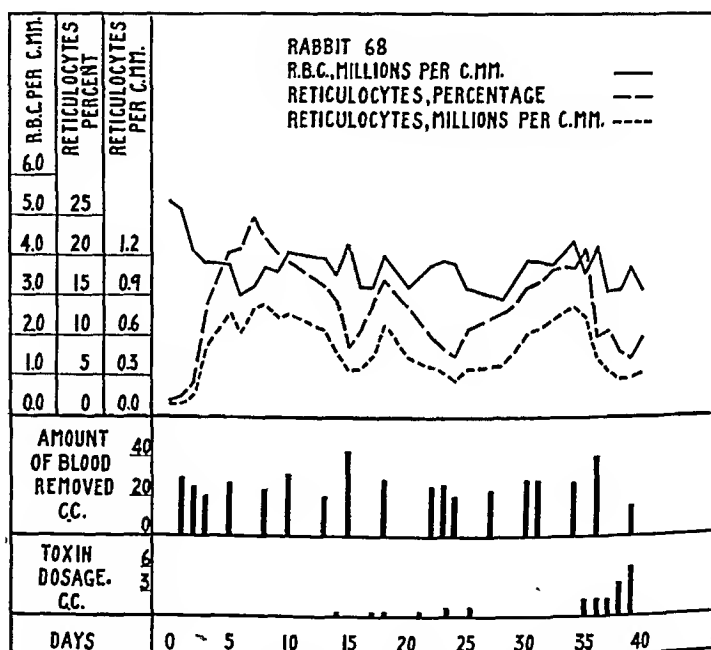


Chart 6.—Observations on the blood of Rabbit 68 injected with *Staphylococcus aureus* hemotoxin.

were transient and not constantly observed. Both animals lost a small amount of weight,  $\frac{1}{4}$  to  $\frac{1}{2}$  pound, during the period of intoxication. Examination of the blood smears showed anisocytosis, polychromatophilia, and occasional normoblasts.

#### COMMENT

The mechanism by which bacterial toxins produce anemia has not been fully explained. In the course of acute bacterial infections, in which the blood stream is invaded, there is increased hemolysis of red blood cells. To an unknown extent such infections are believed also to exert a depressing action on bone marrow activity.<sup>7</sup> In the very common "secondary anemias" associated with chronic infections, the process by which the anemia is produced is more obscure. Yet in such chronic infections as rheumatic fever and chronic osteo-

myehtis there is often a profound anemia At times small, hidden, or apparently unimportant foci of infection may be responsible for an anemia of moderate degree

In this study additional data concerning the origin of this type of anemia have been collected by a study of the peripheral blood In order that minor changes in the marrow activity might be discerned and all variations be brought out more strikingly, the marrow was thrown into a high degree of erythrogeinic activity by repeated bleedings Also, in the presence of a chronic anemia, when there is a constant demand for more red blood cells in the peripheral circulation, young cells are rapidly delivered to the blood By maintaining this want for more cells, the storage of mature cells in the marrow or other organs is precluded, and thus a possible source of error is eliminated

The administration of bacterial toxins to rabbits previously made anemic by bleeding produced a distinct reduction in reticulocyte values in all cases In the intoxicated animals the average daily bleeding required to maintain the red blood cell count at the pretoxin level was reduced in every instance It is obvious that the toxins entered into the picture as etiologic factors in the maintenance of the anemia If the action of toxin had been primarily that of internal blood destruction, then reticulocytosis should have been further stimulated, just as additional bleeding adds an extra impetus to blood formation, but this was not true

The red bone marrow of the animals that were examined at necropsy was definitely hyperplastic Histologic examination showed the tissue to be highly cellular, with an apparent increase in the proportion of erythroid cells, and the sinusoids dilated and engorged The absence of evidence of unusual erythrophagocytosis in the spleen sections suggested that erythrocytes were not being destroyed in excessive amounts This seemed to indicate that the failure to maintain the red blood cells in the peripheral blood at the pretoxin level was due to factors relating to the bone marrow Declining reticulocyte counts with associated reductions in the amount of daily bleeding in the presence of marrow hyperplasia suggested that the mechanism was an interference with the maturation of cells and their delivery by the bone marrow to the peripheral blood Significant changes in the marrow activity, as revealed by a study of the peripheral blood, are shown in Table I

TABLE I

AVERAGE RETICULOCYTE VALUES AND BLEEDING DATA OF RABBITS BEFORE AND AFTER THE INJECTION OF VARIOUS BACTERIAL TOXINS

BACTERIAL TOXIN USED	AVERAGE PRETOXIN RETICULOCYTE VALUE	AVERAGE TERMINAL RETICULOCYTE VALUE	AVERAGE DAILY BLEEDING ANEMIC CON- TROL PERIOD	AVERAGE DAILY BLEEDING PERIOD OF INTOXICATION
	Millions per c mm	Millions per c mm	cc	cc
Tetanus toxin				
1 Lethal amount	0.72	0.14	20.3	10.8
2 Sublethal amount	0.72	0.73	16.8	14.5
Diphtheria toxin	0.69	0.21	14.6	9.5
	0.85	0.76	17.8	14.9
	0.87	0.38	16.4	7.6
	0.53	0.36	13.4	7.8

Other factors have been shown to inhibit marrow activity. In a series of anemias of varied etiology, Rich<sup>8</sup> showed that large transfusions were followed by a drop in reticulocytes in the peripheral blood and interpreted this change as probably due to a decrease in the normal stimulus for marrow activity. Smithburn and Zervas<sup>9</sup> have pointed out that during the period of hyperpyrexia in a bacterial infection there was a marked reduction in reticulocytosis. In a study of the anemia which occurs in cases of chronic nitrogen retention, Parsons and Ekola-Strolberg<sup>10</sup> concluded that the anemia was produced by depression of bone marrow activity. The effect of fever alone upon reticulocyte production and the effects of foreign protein shock and the phenomenon of hypersensitivity on marrow activity have not yet been explained.

#### SUMMARY

Representative bacterial toxins were administered to rabbits in which a high degree of erythrogenic activity had been produced by chronic hemorrhage. The red blood cell count and hemoglobin value were maintained at fairly constant levels by regulating the degree of hemorrhage.

Study of the peripheral blood revealed in all cases that bacterial intoxication produces a definite but variable reduction in reticulocyte values. Standard tetanus and diphtheria toxins in lethal doses markedly interfered with normal marrow activity. Sublethal doses produced temporary changes. Diphtheria toxoid caused little effect. Streptococcus and staphylococcus hemotoxins produced definite but milder interference with erythropoiesis.

In the period of intoxication, the average daily bleeding required to maintain the pret toxin anemic level was reduced in all cases. The absence of evidence of excessive red blood cell destruction pointed to disturbed bone marrow activity. Evidence presented here indicates that interference with normal marrow activity is an important factor in the production of anemias by bacterial toxins and suggests that the mechanism is an inhibition of maturation of the erythrocytes in the bone marrow, with consequent failure of delivery of adequate numbers of cells to the peripheral blood.

605 COMMONWEALTH AVENUE

#### REFERENCES

1. Haden, R. L.: Clinical Factors in Production of Anemia and Regeneration of Erythrocytes and Hemoglobin, *M. Clin. North America* 17: 887, 1934.
2. Sabin, F. R.: Bone Marrow, *Physiol. Rev.* 8: 191, 1928.
3. Osgood, E. E., and Haskins, H. D.: Causes, Classification and Differential Diagnosis of Anemias; Based on Detailed Examination of Over 200 Patients and Study of Literature, *Ann. Int. Med.* 5: 1367, 1932.
4. Steele, B. F.: Effects of Blood Loss and Blood Destruction Upon Erythroid Cells in Bone Marrow of Rabbits, *J. Exper. Med.* 57: 881, 1933.
5. Price Jones, Cecil: Observations on the Changes Produced in the Blood and Bone Marrow by Haemorrhage and Blood Destruction, *J. Path. & Bact.* 15: 4, 1910; 16: 48, 1911.
6. Muir, Robert, and M'Nee, J. W.: The Anaemia Produced by a Haemolytic Serum, *J. Path. & Bact.* 16: 410, 1911.
7. Castle, W. B., and Minot, G. R.: Pathological Physiology and Clinical Description of the Anemias, New York, Oxford Loose-Leaf Medicine 30: 205, 1936.
8. Reich, Carl: Effect of Blood Transfusions on Bone-Marrow Activity as Indicated by Reticulocyte Count, *Am. J. M. Sc.* 182: 513, 1931.
9. Smithburn, K. C., and Zervas, L. G.: Inhibitory Action of Infection and Fever on Hematopoietic Response in Case of Pernicious Anemia, *Ann. Int. Med.* 4: 1165, 1931.
10. Parsons, L., and Ekola-Strolberg, M.: Anemia in Azotemia, *Am. J. M. Sc.* 185: 151, 1933.

## AN ATTEMPT TO MOBILIZE LIPOIDS FROM STORAGE DEPOTS BY DEEP MASSAGE AND INCREASED TISSUE TEMPERATURES\*

JAMES J. SHORT, M.D., AND JOHN DEPAUL CURRANCE, M.D.  
New York, N. Y.

THE object of this study was to determine whether or not fats could be mobilized from their depots by mechanical or thermal means. Heat and massage have been employed for reduction purposes from time immemorial. Claims for local removal of fat by massage are often made. If it be possible to mobilize fat by this means, would lipoids appear in the blood stream in sufficient quantities to be detectable by quantitative chemical analysis?

Body lipoids are usually classified under three headings: (a) true fats, or triglycerides of fatty acids, (b) lipids or phospholipids combinations of fatty acids with phosphorus, either with or without nitrogen, (c) nonsaponifiable substances of somewhat similar nature, such as the sterols of which cholesterol is the chief representative in the animal organism. Distinctions are sometimes made between the tissue fat and the storage or depot fat for which there is some justification. Fats are readily mobilized from the storage depots in response to a demand for fuel. In extreme emaciation, however, a certain amount still remains in the tissues. This tissue fat has been called by the French the "élément constant" and has been found to consist of phospholipids. This does not mean that phospholipids are unavailable for heat and energy, but depot fat seems to be more readily utilizable for this purpose.

Human depot fat is composed largely of stearin, palmitin, and olein. The first two are fully saturated and are solid at body temperature. Olein is unsaturated and liquid at even less than body temperature. This mixture of depot fats is liquid in the body because of the high proportion of olein which comprises approximately 75 per cent of the mixture. Stearin is present only in a negligible amount, while palmitin is present to the extent of about 20 per cent.

Just how fats are mobilized to the tissues is somewhat obscure. In the intestines they are emulsified by the bile, and split by the lipase of the pancreatic secretion and the succus entericus. They are then absorbed into the lacteals and carried to the thoracic duct through which they obtain entrance to the blood stream. Blood fatty acids begin to increase about two hours after a meal and reach a peak about three to five hours later, when they then decline. There is evidence that the phospholipids and sterols play a role in the resynthesis of neutral fat, and in its mobilization and deposition in the depots. There is further evidence that free fat can enter the fat cells of the reserve depots unchanged,

\*From the Departments of Medicine and Physical Therapy, New York Post Graduate Hospital.

Received for publication May 21 1938

TABLE I

Effect of Deep Massage Upon Fatty Acids and Cholesterol of the Blood in Obesity. One Half Hour Deep Massage Over Obese Areas.

CASE NO.	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR -	BEFORE	AFTER	PER CENT + OR -
1	362	391	+ 8.0	176	193	+ 9.7
2	384	376	- 2.1	240	230	- 4.2
3	446	421	- 5.6	187	193	+ 3.2
4	397	441	+11.0	181	200	+11.0
5	353	401	+13.6	207	207	0.0
6	411	459	+11.8	183	188	+ 2.7
7	402	390	- 3.0	236	172	-27.2
8	668	691	+ 3.4	274	264	- 3.8
9	361	330	- 8.6	182	176	- 3.3
10	307	365	+18.5	176	166	- 5.7
11	365	313	-14.2	150	162	+ 8.0
12	218	200	- 8.3	176	193	+ 9.7
13	224	227	+ 1.3	240	272	+13.3
14	341	354	+ 3.8	215	222	+ 3.3
15	409	414	+ 1.2	187	200	+ 6.9
16	388	367	- 5.4	193	208	+ 7.8
17	270	284	+ 5.2	162	166	+ 2.5
18	378	361	- 4.6	200	206	+ 3.0
19	362	390	+ 7.8	230	222	- 3.5
20	498	504	+ 1.2	240	230	- 4.2
Average	377	384	+ 1.8	202	204	+ 1.0

TABLE II

Effect of Local Heat Upon Fatty Acids and Cholesterol of the Blood in Obesity. Areas Heated by Means of Induction Coils and Short Wave Therapy.

CASE NO.	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR -	BEFORE	AFTER	PER CENT + OR -
21	485	555	+14.4	300	300	0.0
22	385	339	-11.9	240	200	-16.6
23	457	585	+28.1	260	214	-17.7
24	505	487	- 3.6	231	240	+ 3.9
25	407	418	+ 2.7	230	222	- 3.4
26	390	404	+ 3.6	206	222	+ 7.8
Average	438	465	+ 6.2	245	233	- 4.9

but to gain access to the tissue cells, such as the liver, the fatty acids must first be converted to a phosphatid by the addition of phosphorus and nitrogen.

In a former study, the writer demonstrated an increase in the total blood fats following ether anesthesia.<sup>1</sup> This was attributed to the solvent effect of ether and its ability to carry fat with it into the blood stream. Murlin and Riche<sup>2</sup> reported an increase in blood fats after exercise in 1916. In our study active exercise was not used, but merely vigorous heavy massage for one-half to one hour over areas like the thighs and hips of obese individuals where fat was particularly in evidence. With the patient in the postabsorptive state, blood was taken before and immediately following the massage or thermal treatment. It was then analyzed for total fatty acids and cholesterol. Total acetone bodies were done on the early experiments, but were discontinued because they were uniformly within normal limits. The findings for fatty acids and cholesterol are shown in Tables I-IV.

TABLE III

Effect of Local Heat Followed by Deep Massage Upon Fatty Acids and Cholesterol of the Blood in Obesity Heat by Induction Coil

CASE NO	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR -	BEFORE	AFTER	PER CENT + OR -
27	442	460	+ 4.6	187	167	-10.7
28	387	358	7.5	250	230	- 8.0
29	403	390	3.2	260	273	+ 5.0
30	401	331	17.5	240	230	- 4.2
Average	408	385	5.9	254	225	- 3.8

TABLE IV

Effect of Artificial Fever Upon Fatty Acids and Cholesterol of the Blood in Obesity Blood Taken After Approximately Thirty Minutes' Immersion in Tank of Hot Water With Oral Temperature at 101° F

CASE NO	FATTY ACIDS			CHOLESTEROL		
	BEFORE	AFTER	PER CENT + OR -	BEFORE	AFTER	PER CENT + OR -
31	335	390	+15.4	213	222	+ 3.9
32	329	400	+21.6	230	240	+ 4.4
33	382	375	1.8	250	230	- 8.0
34	397	404	+ 1.8	171	174	+ 1.7
35	384	388	+ 1.0	187	187	0.0
36	347	339	- 2.3	193	181	- 6.2
37	438	422	- 3.7	300	260	- 13.3
Average	373	388	+ 4.1	221	219	- 0.9

## COMMENT

There is some obscurity about the means by which fats are mobilized from their depots from the production of heat and energy. It seems reasonable to believe that mechanical and thermal means, such as were used in these experiments, might cause a sufficient added increment to the blood stream to be detectable by chemical analysis. This was not proved to be the case. Several explanations of our negative findings suggest themselves: (a) lipoids cannot be mobilized from their depots by the means employed, (b) the amount mobilized was too small to be detectable when added to the relatively large volume of circulating blood, (c) a sufficient time interval was not allowed to permit the lipoids to reach the blood in greatest concentration, (d) fluids were simultaneously mobilized from the tissues which, by blood dilution, obscured the added increment of lipoids. Further studies will be necessary to determine which explanation is correct.

## REFERENCES

- 1 Short, J. J. The Formation of Acetone Bodies Following Ether Anesthesia and Their Relation to the Plasma Bicarbonate, *J Biol Chem* 41: 503, 1920.
- 2 Murlin, J. R., and Riche, J. A. The Fats of the Blood in Relation to Heat Production, Narcosis, and Muscular Work, *Proc Am Physiol Soc* 40: 146, 1916.



## SKIN REACTIONS\*

### VI. A SIMPLE MICROMETHOD FOR THE ASSAY OF HISTAMINE IN MAMMALIAN BLOOD

HAROLD A. ABRAMSON, M.D., AND I. OCHS, A.B.  
NEW YORK, N. Y.

#### INTRODUCTION

IN THE absence of high concentration of electrolytes, histamine in dilutions as high as 1:5,000,000 may be detected by wheal formation when forced into the human skin by means of the direct electrical current.<sup>1</sup> That is, if some absorbent material is wet with a solution of histamine and made the positive pole by means of a suitable metal contact, at comparatively low current densities sufficient histamine is carried into the skin by ionic and electro-osmotic transport to produce its characteristic effect. In view of the difficulties attendant both upon the chemical determination of histamine and upon biological assay by means of the contraction of smooth muscle, a simple method to detect histamine in the presence of blood would be desirable. This method, based upon the response of the skin by wheal formation subsequent to electrical transport of histamine into the skin, has now been developed and forms the subject of this communication.

#### METHOD

*Source of Current.*—The apparatus previously described by Abramson and Alley<sup>1</sup> may be used if it is desired to make more than one determination at a time. The equipment consists merely of a 45 volt B battery which can be tapped through one or more potentiometers. Milliammeters reading from 0 to 5 Ma. are in series in the circuit of the subject.

*Electrode Materials.*—The absorbent material containing the solution suspected of having histamine is placed in contact with the skin of the anterior aspect of the forearm, a metal electrode being employed to establish contact with the source of current. The success of the method depends upon its reproducibility; and the reproducibility in high dilutions depends largely upon the choice of the material for the electrodes. After some experimentation, the copper gauze electrode described by Abramson and Alley was discarded in favor of a electrode of light copper foil, curved slightly so that it rested evenly on the absorbent electrode. The pressure was thus distributed uniformly because the foil is thin enough to yield to pressure before the skin itself is appreciably distorted. Pressure on the developing wheal may retard or prevent its appearance.

\*From the Biological Laboratory, Cold Spring Harbor, the Medical Service of Dr. George Baehr, and Laboratories of the Mount Sinai Hospital, New York.  
Received for publication, May 26, 1938.

The following absorbent materials were tested absorbent cotton, Canton flannel, blotting paper, and asbestos paper. Electrodes (having an area of 1 sq cm) of these materials were examined with special reference to their usefulness at low concentrations. Although the blotting paper is advantageous as far as uniformity and ease of handling are concerned better distribution of pressure and electrical contact was obtained with absorbent cotton. Surprisingly small volumes of liquid are needed to wet 1 sq cm of cotton or blotting paper between 1 to 2 mm thick. The final technique employed required 0.1 cc of liquid for each determination, although smaller volumes of liquid could be used if the area of the electrodes was correspondingly reduced. Whatever absorbent material is used, however must be tested for the presence of contaminating (wheal producing) substances. Observations were usually made at the end of ten or fifteen minutes, although the wheals were frequently observed for much longer periods. The investigator will find it desirable to standardize his own technique. Thus, with high dilutions it may be necessary to observe the maximum effect, rather than the effect at a given time.

*Position of Negative Electrode*—The effect of the position of the negative electrode on the manner in which the wheal spread was studied in the following way. The negative electrode was either held in the hand or placed immediately above, below, or opposite the positive electrode, with no obvious influence on the direction of the spread of the wheal. In the experiments reported here, the negative electrode was usually held in the hand of the same arm used.

It was also of interest to see if the extent of pseudopod formation depended primarily upon the distribution of the lines of electric current. Duco house hold cement painted on the skin acts as an efficient insulator and prevents wheal formation following histamine iontophoresis over a protected area. The film of cement is sufficiently transparent to observe many phenomena occurring beneath it. Histamine wheals were produced on the anterior surface of the forearm, with frames of Duco cement about the absorbent cotton. In this way the lines of current entering the skin could not pass through the Duco frame. Wheals produced under these conditions developed pseudopods which could be seen to spread beyond the inner boundary of the insulating frame, and extended widely beyond it, with higher concentrations of histamine. In other words, the spread of the wheal was independent of the lines of current. These as well as similar experiments, lend support to the point of view expressed by Abramson and Engel,<sup>2</sup> following the observation of McMaster and Hudack, that wheal formation of this type is due essentially to a convection of histamine through the lymphatic capillaries with escape of histamine back into the interstitial tissues to affect the blood capillaries locally at the site of escape.

*Limits of the Method*—Since the detection of the presence and quantity of histamine by the method of iontophoresis is a biological assay, the absolute quantity of wheal producing substance cannot be measured. However, the method does not suffer from the restriction imposed by the destructive processes of chemical procedures necessary in the chemical methods. Extracts of blood having activities like that of histamine have been extensively investigated. Although Harris<sup>3</sup> employed simple alcoholic extracts, Best and McHenry<sup>4</sup> boiled the blood with hydrochloric acid. Code has maintained that boiling with acid

might lead to secondary reactions. He developed methods free from this objection. As far as we have been able to discover, assays have previously been performed by observing the effects of extracts on the contraction of smooth muscle. In the absence of electrolytes, about one part in five million of histamine can usually be easily detected by the appearance of scattered papules on the skin of the forearm. Mammalian blood is approximately equal in electrolyte content to M/7 sodium chloride solution. As pointed out by Abramson and Alley,<sup>1</sup> salts may, and in sufficient quantity do, diminish the histamine transported not only because of a partition of current carried between positively charged histamine and other ions, but also because of the depressing effect of salts on the electrokinetic potential of the skin. For this reason, the lowest concentration of histamine which can be detected lies between 1:2,000,000 and 1:5,000,000.

TABLE I

WHEEL PRODUCTION BY DEFIBRINATED BLOOD CONTAINING VARIOUS QUANTITIES OF HISTAMINE (UNWASHED ABSORBENT COTTON)

HISTAMINE CONCENTRATION	RESULT
0	No wheal
1:10,000,000	No wheal
1:1,000,000	Flat, fairly confluent wheal
1:1,000	Spreading wheal with pseudopods

## EXPERIMENTAL

*Recovery of Histamine From Human Blood.*—Preliminary experiments with human blood obtained from pricking the finger tip showed that capillary blood did not contain substances which produced a wheal with our technique. The detection of histamine in defibrinated blood is given in Table I. The data in the table were obtained by making dilutions of histamine in freshly defibrinated blood. With 1:1,000,000 histamine, in the presence of blood, a well-defined, fairly confluent wheal was obtained under the conditions described in Table I. This typical experiment also disclosed that insufficient whealing substance formed in the blood standing at room temperature after one hour. Further, histamine may readily be detected almost quantitatively if present in human blood in the concentrations described, for the type of wheal obtained corresponds approximately to that usually observed with simple solutions of histamine having similar quantities of electrolytes.

In addition to the experiment described in the foregoing, the following samples of blood or materials derived from blood (one series of experiments) did not contain sufficient whealing substance to produce a wheal with our technique. Cotton electrodes and a current density of 0.5 Ma. for five minutes were used.

- (1) Oxalated human plasma.
- (2) Buffy coat from human plasma.
- (3) Buffy coat frozen and then thawed.
- (4) Red cells (washed) following freezing and thawing until hemolyzed.

If these experiments are subsequently confirmed, the histamine liberated and remaining in the solution during the freezing and thawing of human blood cells is probably less than one part in three million.

*Recovery of Histamine From Rabbit Blood.*—The whole blood of the rabbit apparently contains a substance capable of being transported electrically

to produce a wheal in normal human skin.<sup>3</sup> In 18 experiments with whole rabbit blood, using 8 different rabbits in 5 different individuals apparently not sensitive to rabbit serum, definite wheals were obtained in 16 instances under conditions identical with those producing no effect with saline or human blood. If sensitiveness of these individuals to rabbit blood itself can be excluded, the production of a wheal under these circumstances is confirmatory evidence of the data of Codes that normal rabbit blood contains approximately one part in one million of histamine or of a histamine like substance. In all of the instances in which wheals were produced by rabbit blood, a 1:10 dilution of the blood with saline resulted in a complete loss of the wheal producing properties of the solution when administered by the method in question. It was concluded from these experiments that the study of the effects of blood on the wheal producing properties of histamine might be investigated under the conditions described in the foregoing if precautions were taken to dilute the blood to one part in ten of saline.

Histamine was dissolved in distilled water and 0.1 c.c. of the solution was added to cotton squares. These were dried for one and one half hours in an oven at 60° C and then five hours in a calcium chloride desiccator. The effect of whole, nonoxalated, rabbit blood on these cotton squares containing varying concentrations of histamine is given below.

TABLE II

HISTAMINE CONCENTRATION	RESULT
0, 1 part blood, 10 parts saline	No wheal
0	Fairly confluent wheal lasting about thirty minutes
1:5,000,000	Fairly confluent wheal lasting about thirty minutes
1:500,000	Fairly confluent wheal lasting one hour, slight spread
1:150,000	Large, fairly confluent wheal lasting two and one half hours marked spread with many pseudopods

TABLE III

WHEAL FORMATION BY RABBIT'S BLOOD (DILUTED 1:10 WITH PHYSIOLOGIC SALINE) CONTAINING VARIOUS QUANTITIES OF HISTAMINE

HISTAMINE CONCENTRATION	RESULT
0	No wheal
1:4,500,000	Small discrete papules
1:1,500,000	Fairly confluent flat papules
1:150,000	Well marked spreading wheal

Although whole rabbit blood contains a wheal producing substance, histamine was readily detected (a) when histamine was added to rabbit's blood, (b) after rabbit's blood containing histamine was diluted with saline, and (c) in rabbit's blood following intravenous administration of histamine.

(a) To illustrate the first point note the data in Table II which were obtained by adding whole rabbit's blood (nonoxalated) to dry squares of cotton (1 cm.<sup>2</sup>) containing various quantities of histamine. These were prepared by adding 0.1 c.c. of histamine solutions of various concentrations to cotton squares

(1 cm.<sup>2</sup>) and drying the cotton at 60° C. for one and one-half hours and in a desiccator for five hours. Blood was added previous to each experiment.

(b) If oxalated rabbit blood is added to saline containing histamine and the mixture is diluted with saline to higher dilutions, the histamine can be readily assayed. An experiment of this type is depicted in Table III. The initial dilution of blood was 1:10 and that of the histamine 1:10,000. The first dilution of the blood resulted in removing the effective wheal-producing substance. Subsequent dilution by saline permitted the whealing effects to be observed in dilutions up to about 1:4,000,000.

(c) That histamine may be readily detected in rabbit's blood is perhaps demonstrated more clearly by the following experiment. A rabbit, weighing 2 kg., was given 2 mg. of histamine intravenously by a marginal vein of the ear. While the animal was dying, blood was obtained by cardiac puncture. Before the injection of histamine, assay of a 1:10 dilution of blood in saline produced no wheal as usual. After histamine, the following results were obtained (current density 0.5 Ma. for five minutes):

DILUTION OF BLOOD	CHARACTER OF WHEAL
1:10	Irregular confluent wheal lasting thirty-five minutes
1:100	Slight, faint, discrete papules
1:1000	No wheal

According to this assay, the papules observed with a 1:100 dilution of blood represented a dilution of histamine close to 1:5,000,000. On this basis the dilution in the blood was 1:50,000. Indeed, the undiluted blood of the animal in question after the administration of histamine did give a large, persistent, confluent, spreading wheal by iontophoresis. If the blood volume of the rabbit is taken as 200 c.c., the immediate dilution (death occurred within two minutes) would be 1:50,000, which corresponds to that dilution observed in the assay.

In another rabbit, which was killed by 0.7 mg. of histamine intravenously, the blood obtained by cardiac puncture produced minute, discrete papules in a 1:10 dilution in saline after five minutes, with a current density of 0.5 Ma. The 1:100 dilution was negative. If the blood volume of the rabbit was 200 c.c., the immediate dilution of the histamine in the blood was about 7:2,000,000 or 1:3,000,000, approximately. The size and type of wheal observed was roughly in agreement with this calculation.

TABLE IV  
HISTAMINE ADDED TO GUINEA PIG BLOOD AND DILUTED WITH SALINE

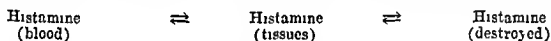
HISTAMINE CON- CENTRATION	RESULT	
	FIVE MIN.	TEN MIN.
0	No whealing	No whealing
1:4,500,000	Scattered discrete, slightly elevated papules	Scattered discrete, slightly elevated papules
1:900,000	Confluent central wheal surrounded by discrete papules	Tendency toward confluence and somewhat flatter
1:100,000	Confluent wheal larger than area of the electrode; discrete papules on periphery	Confluent flat wheal

*Recovery From Guinea Pig Blood*—Ovalated blood from two adult guinea pigs by heart puncture did not produce wheals with our method. Guinea pig blood (ovalated) did not prevent the histamine from exercising its usual whealing effect. Further, histamine was also readily detected if guinea pig blood containing histamine was diluted with isotonic saline (Table IV).

To determine if histamine could be detected in guinea pig blood following intraperitoneal administration, 48 mg of histamine was given to a guinea pig weighing 580 gm. While the animal was dying, blood was withdrawn by cardiac puncture. The ovalated blood (which on two previous occasions showed no whealing), now produced fine discrete papules, corresponding to about 1 5,000,000 histamine. The 1:10 dilution produced no wheal. In a similar experiment which differed from the preceding, the guinea pig was practically symptom free fifteen minutes after the injection and subsequently recovered. There was no whealing with the ovalated blood. According to our assay, the dilution of histamine in the blood was less than 1 5,000,000 in this instance.

#### DISCUSSION

Since the blood of the rabbit killed by less than 1 mg of histamine given intravenously contained considerable quantities of histamine, it follows that only a small part of the histamine originally administered was needed to produce death. That is, an equilibrium of the following type may be set up:



This equilibrium would exist in favor of the first term according to the experiment just cited. Unpublished data obtained with Lubkin during a study of the histamine wheal indicated that following wheal formation by iontophoresis of histamine, it can be recovered by reversed iontophoresis for as long as forty minutes under certain conditions.<sup>6</sup> The type of equilibrium in the dermis analogous to the preceding has been studied and will be described in a subsequent communication.

With the technique described in the foregoing the skin of most subjects responded by discrete papules to a concentration of histamine close to 1 5,000,000. The marked differences in sensitiveness to histamine supposed to exist for the skin surfaces of different individuals were not noted. Our impression is that there was more variation in response to the electric current alone (controls) than to the histamine administered. The erythema produced by the current alone was usually fairly well localized to the electrode area, whereas that produced by histamine was always extended radially from it.

Horsfall<sup>7</sup> has observed a phenomenon closely related to the observation that wheals can be produced by the iontophoresis of rabbit blood. Horsfall found that intradermal injections of rabbit antipneumococcus serum was followed by whealing in individuals not immunologically sensitive to rabbit serum. Even though the skin test may be positive to these sera, they may be administered intravenously without anaphylactic responses. Since we have shown that the wheal producing agent is readily introduced into the skin by electrophoresis from the positive pole, it could be inferred that the whealing substance is positively charged. However, this is by no means a necessary conclusion. As noted

hitherto, histamine has been transported electrically into the skin even when it was dissolved in aqueous solutions of alkali the pH of which was sufficient to almost completely suppress the ionization of the histamine salt. Under these conditions the undissociated histamine is transported by electro-osmosis. It is of some importance to determine in more detail the nature of the whealing substance in rabbit sera.

There are many body fluids and tissues which are supposed to contain histamine in considerable quantities. The method described here can probably be adapted for the determination of histamine in gastric contents, for extracts of the skin and other tissues. The method would provide, for example, a simple procedure for following the rate of disappearance and fate of histamine in animals like the rat, which are insensitive to massive doses.

#### SUMMARY

1. Previous investigations have shown that wheal formation by histamine following electrical transport into the human skin permits the detection of histamine in dilutions as high as 1:5,000,000.

2. A standard, more reproducible method of applying the positive electrode has been developed.

3. Wheal formation was essentially independent of the position of the negative electrode and of the lines of current at the positive pole.

4. After addition of histamine it was detected in human, rabbit, and guinea pig blood in dilutions between 1:2,000,000 and 1:5,000,000 by the electrophoretic method of producing wheals.

5. Using this method, it was found that rabbit blood itself produced wheals in five individuals. This capacity to produce wheals disappeared when the blood was diluted ten times. Human and guinea pig blood did not contain a wheal-producing substance. Histamine was easily detected in the cardiac blood of rabbits killed by intravenous administration of histamine.

6. The bearing of the data obtained thus far on the nature of the equilibrium between histamine and the tissues is briefly discussed.

We are indebted to Mrs. Margery Engel for assistance in these experiments.

#### REFERENCES

1. Abramson, H. A., and Alley, A.: *Arch. Phys. Therap.* 18: 327, 1937.\*
2. Abramson, H. A., and Engel, M.: *J. Invest. Dermat.* 1: 65, 1938.
3. Harris, K. E.: *Heart* 14: 161, 1927.
4. Best, C. H., and McHenry, E. W.: *J. Physiol.* 70: 349, 1930.
5. Code, C. F.: *J. Physiol.* 89: 257, 1937.
6. Code, C. F., and Ing, H. R.: *J. Physiol.* 90: 501, 1937.
7. Abramson, H. A., Engel, M., Lubkin, V., and Oels, I.: *Proc. Soc. Exper. Biol. & Med.* 58: 65, 1938.†
8. Horsfall, F. L., Goodner, K., and Macleod, C. M.: *New York State J. Med.* 38: 1, 1938.

\*Dr. Peyton Rous has informed one of us (H. A. A.) that Ebbecke (*Arch. f. d. ges. Physiol.* 195: 300, 1922) was the first to employ histamine iontophoresis.

†One of us (H. A. A.), working with Gorin and Engel, found that histamine may be recovered from the skin for at least two days subsequent to histamine iontophoresis. Its presence in the skin may frequently be shown for four days. This was not so following ordinary intradermal administration.

# LABORATORY METHODS

---

## A METHYLENE BLUE STAIN FOR SECTIONS OF FORMALIN FIXED MARROW

---

HAROLD GORDON, M S, M D, LOUISVILLE, KY

---

THE following methylene blue stain is recommended for sections of formalin fixed bone marrow. The method is simple, rapid, and gives constant results. It depends upon the simple principles of deformolization, sensitization, over staining, and selective differentiation.

### METHOD

Suitable pieces of bone marrow are fixed in 10 per cent formalin solution (4 per cent formaldehyde), decalcified in dilute mineral acid, washed in water, hardened and dehydrated in alcohol, cleared in xylol, and embedded in paraffin. In this laboratory 20 per cent nitric acid is used as the decalcifying agent, and the calcium salts are removed under vacuum. This reduces the time needed for decalcification, keeps the tissue in motion, and inhibits the formation of large bubbles of carbon dioxide gas which damage the tissues. Alternatively the bone may be decalcified in the sodium citrate, formic acid mixture suggested by Evans and Kiaian<sup>1</sup>. Sections should be cut 4 to 6 microns thick, affixed to cover slips or slides, and dried in the oven. If the glassware is thoroughly clean and free of grease, the sections will adhere to the glass without egg albumen. The paraffin is removed with xylol, and the sections are brought into water in the usual manner.

*Deformolization*—The formalin fixative is removed by immersing the sections for one minute in 10 per cent ammonia solution (one part of 28 per cent ammonia and nine parts of water) kept in a covered dish. Longer immersion is undesirable because the sections may become detached. The ammonia gradually loses its strength and should be renewed as required. (Zenker fixed material may be used. It does not require the ammonia bath, instead, it should be treated with Lugol's iodine, followed by sodium thiosulfate solution and a thorough wash in water to remove the precipitates of mercury salts.)

*Sensitization*—The sections are washed in water to remove the ammonia and mordanted for three minutes in a mixture consisting of equal parts of Zenker's fluid (without acetic acid) and saturated aqueous solution of copper sulfate.

*Staining*—After a thorough wash in two changes of water, the sections are stained for one and a half to three minutes in the following mixture

---

1 per cent aqueous solution of eosin Y	one part
1 per cent aqueous solution of phloxin	three parts

\*From the Department of Pathology School of Medicine University of Louisville  
Received for publication April 6 1938



They are then washed in water and stained for four minutes in Loeffler's alkaline methylene blue, heated to 37° C.

*Differentiation.*—The sections are washed in water and differentiated in absolute alcohol. Differentiation should be controlled by microscopic examination. As soon as differentiation is complete, the sections are cleared in xylol and mounted in balsam.

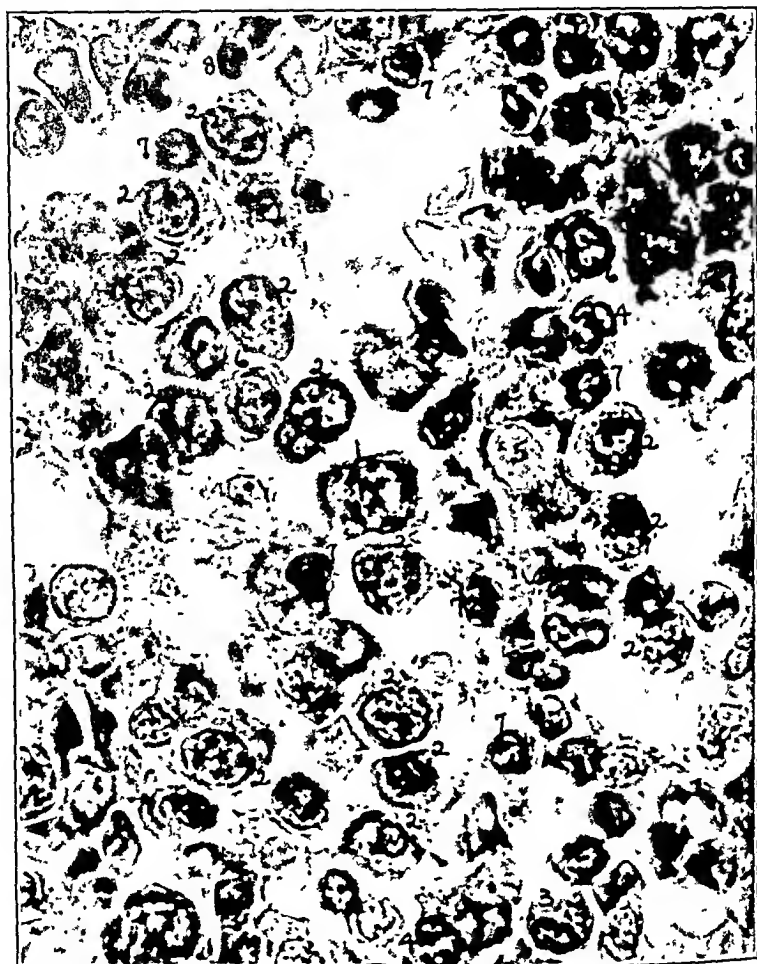


Fig. 1.—Section of sternal marrow from a case of myelogenous leucemia. Tissue was fixed in formalin, decalcified with dilute nitric acid, and stained with methylene blue, eosinophloxin. Magnification 740 diameters. 1, premyelocyte; 2, neutrophilic myelocytes; 2a, dividing myelocytes; 2b, eosinophilic myelocytes; 3, metamyelocytes; 4, young polymorphonuclear leucocytes; 5, endothelial cells; 6, macronormoblasts; 7, intermediate normoblasts; and 8, micronormoblasts. A majority of the cells are myelocytes, but there are also a number of normoblasts.

**Results:** The marrow cells, stroma, and capillaries are all stained very precisely (Fig. 1). The granules of the myeloid cells stand out sharply. The normoblasts have a faint red cytoplasmic border; the immature cells a pale blue cytoplasm. The nuclei of all cells are sharply outlined in varying shades of blue, so that identification of the various cells is greatly simplified. This is an important consideration now that diagnosis of biopsy specimens of marrow is requested so frequently.

**Comment** The stain does not fade if kept away from strong daylight. Exposure to direct sunlight causes the methylene blue stain to fade completely at the end of two weeks, but the light of a 15 Watt substage microscope lamp has no visible effect at the end of six weeks' continuous exposure. While primarily devised for sections of bone marrow, the method is equally suitable for soft tissues.

The time periods advised give optimum results but may be varied. Differentiation in alcohol is the only step requiring careful attention. With a

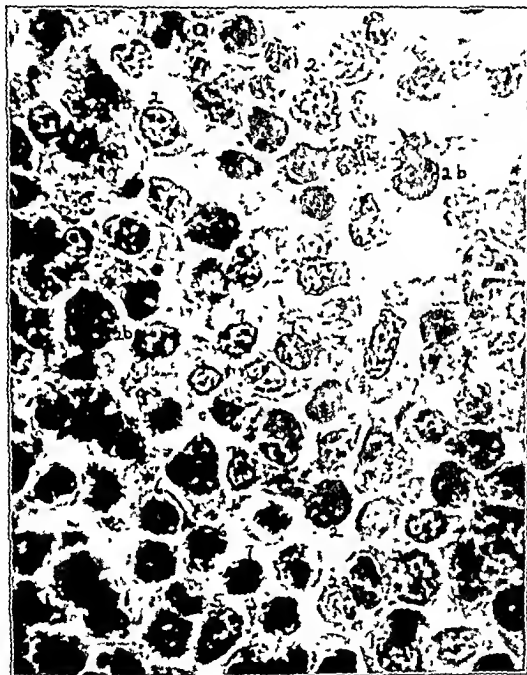


Fig. 2—Another field from the same section showing two dividing cells and the coarse granules of eosinophilic myelocytes.

little experience, however, the correct stage at which differentiation should be arrested may be recognized by the unaided eye. The methylene blue is removed slowly by absolute alcohol (much more rapidly by 95 per cent alcohol), while the cytoplasmic stain is alcohol fast. Neither eosin nor phloxin—alone or in succession—gives as bright or precise a stain as the eosin-phloxin mixture. This mixture keeps indefinitely and may be used repeatedly. Similarly, copper sulfate by itself is only a fair mordant and its effectiveness is greatly increased when it is combined with Zenker's fluid.

This mixture also may be used repeatedly. Occasionally the sections become loose in the ammonia bath. This may be prevented by floating the sections on to a warm 0.25 per cent aqueous solution of gelatin, as they are cut. They are then transferred to slides or cover slips and dried in the oven in which is kept a small unstoppered bottle of full strength formaldehyde.

#### SUMMARY

A methylene blue stain, suitable for sections of formalin-fixed bone marrow (or soft tissue), is described.

The method may be summarized as follows:

1. Fix tissues in 10 per cent formalin, decalcify in dilute mineral acid, wash in water, harden and dehydrate in alcohol, clear in xylol, and embed in paraffin.

2. Cut sections 4 microns thick, transfer to cover slips or slides, dry in the oven, deparaffinize in xylol, dehydrate in graded alcohols, and place in water.

3. Ten per cent ammonia (2.8 per cent ammonia), one minute. (Zenker-fixed material does not require the ammonia bath, but should be treated with Lugol's iodine for five minutes, washed in water, decolorized in 5 per cent sodium thiosulfate, thoroughly washed in water, and then mordanted and stained as described below.)

4. Wash in tap water.

5. Mordant in a solution composed of equal parts of Zenker's fluid (with-out acetic acid) and saturated aqueous solution of copper sulfate, three minutes.

6. Wash in two changes of tap water.

7. Stain for two minutes in an eosin-phloxin mixture:

1 per cent aqueous solution eosin Y	one part
1 per cent aqueous solution phloxin	three parts

8. Wash in tap water.

9. Stain in Loeffler's alkaline methylene blue, heated to 37° C. four minutes.

10. Wash in tap water.

11. Differentiate carefully in absolute alcohol, clear immediately in two changes of xylol, and mount in balsam.

#### REFERENCE

1. Evans, N., and Krajian, A.: New Method of Decalcification, *Arch. Path.* 10: 447, 1930.

# PHOTOELECTRIC RECORDING OF THE PULSE AND OF OTHER OSCILLATORY MOVEMENTS WITH THE ELECTROCARDIOGRAPH\*

A. B. HERTZMAN, PH.D, ST LOUIS, MO.

MARRAZZI<sup>1</sup> called attention in 1935 to the fact that the photoelectric cell could be conveniently used for the detection and recording of movement. He suggested the possibility of wide application. It seemed worth while to extend his suggestion to the recording of the pulse. The technique described below differs in certain essential details from that of Marrazzi

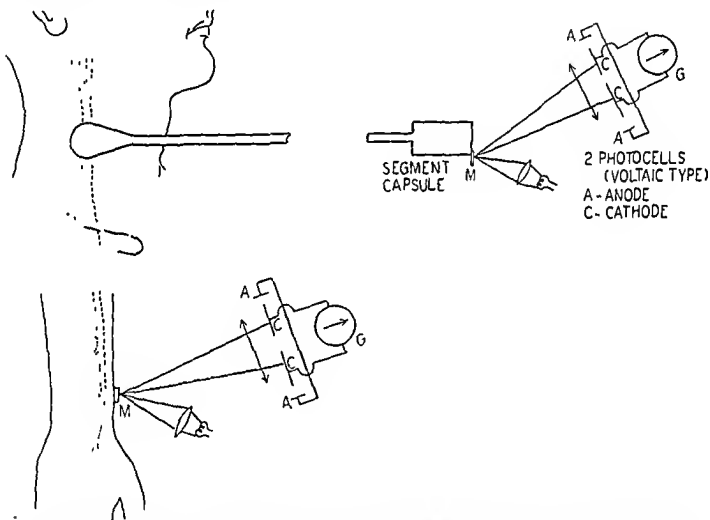


Fig. 1.—Schema of arrangements for photoelectric recording of the pulse with the string galvanometer without amplification. The upper figure shows the recorder using the Frank segment capsule, the lower figure, using a mirror cemented to the skin over the artery.

Two photoelectric cells of the voltaic type (the General Electric Light Sensitive Cells are satisfactory for this purpose) are mounted side by side (Fig. 1). Their cathodes are connected to the lead of the electrocardiograph, while their anodes are connected together. If equal amounts of light fall simultaneously on the two cells, the currents generated in each oppose and nullify

\*From the Department of Physiology, St. Louis University School of Medicine.  
Received for publication, April 13, 1938

each other. This eliminates the need for devices supplying opposing potentials to prevent excessive current outputs by the photocell from reaching the string. If a beam of light is now caused to fall on both cells, its oscillations from one cell toward the other (as indicated by the double arrow in Fig. 1) will be reflected in corresponding current oscillations which are in turn recorded by the electrocardiograph. This fact is made use of in recording the pulse in the manner indicated in Fig. 1, employing either a Frank segment capsule or a mirror cemented to the skin. The light from a penicil type of flashlight or an ophthalmoscope is focused on the mirror and reflected back to the two photocells, so that it falls partly on each. The oscillations of the mirror, with the pulse causing corresponding oscillations of the reflected light beam, are thus recorded by the electrocardiograph. It is unnecessary to control carefully the intensity of the illumination or the distance of the photocells from the mirror, since these things

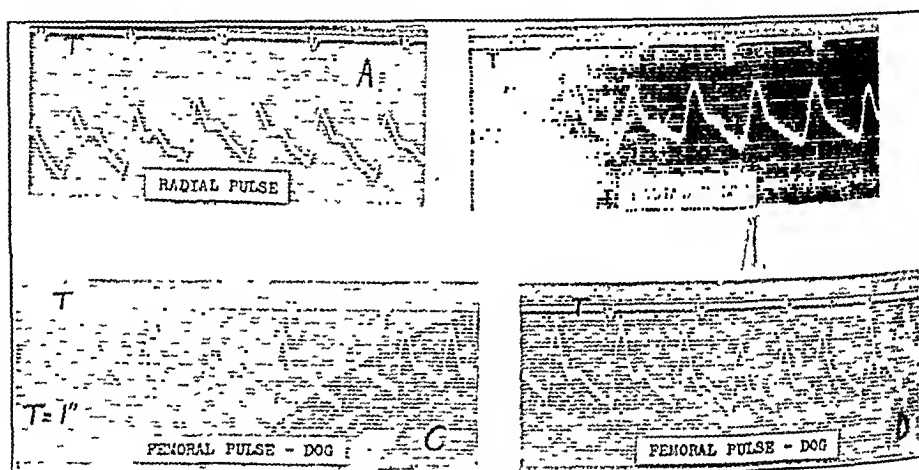


Fig. 2.—The arterial volume pulse recorded photoelectrically with the aid of a mirror cemented to the skin over the artery (*A, B, C*). *A*, young male adult; *B*, male, aged 75 years; *D*, the femoral volume pulse recorded with a photoelectric skin plethysmograph.

affect the two photocells almost equally. The relative amounts of light falling on each photocell are also unimportant. This renders unnecessary particular care in alignment, since the mirror's oscillations are the only important influence causing variations in the photoelectric current delivered to the electrocardiograph.

Illustrative records of the arterial volume pulse so recorded are supplied in Fig. 2. The lower right record taken with a photoelectric skin plethysmograph (the details of which will be reported elsewhere) is inserted for purposes of comparison with the lower left record taken with a mirror cemented to the skin. The essential identity of the two records is apparent.

Variations in the sensitivity of the recording are conveniently provided by the shunts in the string galvanometer or by variations in the intensity of the illumination, thus eliminating the necessity for long optical lever arms. The application to recording of other oscillatory movements, such as those exhibited by optical manometers of the Hamilton or Wiggers type, is obvious.

## SUMMARY

Photoelectric techniques are described which provide in a simple inexpensive manner for direct recording without amplification of the arterial or venous pulses, or of other oscillatory movements by the unmodified electrocardiograph

## REFERENCE

- 1 MARRAZZI, A. S. The Use of the Photoelectric Cell in Physiological Experiments, *Science* 82: 254, 1935

## THE RESPONSE TO LIVER EXTRACT OF EXPERIMENTALLY INDUCED (TYPHOID) ANEMIA IN RABBITS NEGATIVE RESULT\*

ADOLPH J. CRESKOFF, M.D., AND THOMAS FITZ HUGH JR., A.M., M.D.  
PHILADELPHIA, PA.

EXPERIMENTS recently reported by Wolf Weber and Kiogel<sup>1</sup> suggest that anemia induced in rabbits by injections of deproteinized typhoid bouillon concentrate may respond in a specific manner to liver extract, and thus may constitute a method of assaying and standardizing the hematopoietic liver principle. Their "typhoid toxin" is obtained by allowing a typhoid bouillon culture to incubate until "sterile by autolysis" followed by precipitation of the proteins with colloidal iron and concentration of the filtrate *in vacuo*. This material is then injected subcutaneously into rabbits, the effective daily dose being from 15 to 20 cc per kg. of body weight. Because the toxin is an apparently painful irritant, preliminary local anesthesia (novocain) is recommended. Prompt anemia with depression of leucocytes, platelets, and reticulocytes is said to follow daily injections of the toxin. Recovery on discontinuing injections is slow and imperfect, the concomitant reticulocyte response is irregular, low in magnitude, and variable in type. Death frequently ensues before severe anemia develops.

According to these authors if the rabbits are treated beforehand or concomitantly with daily parenteral liver extract, a different result is obtained. Anemia still appears, but the animals invariably survive and, despite continued toxin (and liver) administration, a reticulocyte crisis (34 per cent in one experiment) is said to occur with subsequent recovery of erythrocyte, hemoglobin, leucocyte, and platelet values. The reticulocyte crisis is said to reach a peak between the eighth and twelfth days. Its similarity in time and character to the classical response to specific treatment of human pernicious anemia is stressed. The authors exhibit three graphs as typifying many similar experiments using various brands of commercial liver extract.

A dependable laboratory method of assaying hematopoietic liver substance has long been a clinical and commercial desideratum. Many attempts have been made, but all have proved either impractical or unreliable. A tiny

\*From the Hematological Section of the Medical Clinic Hospital of the University of Pennsylvania. This work was aided by grants to the Hematology Fund from Mrs. Bruce Ford and Mr. Richard P. Brown.

specific method of bioassay would on a priori grounds seem to prediate the production in the experimental animal of a pathologic state presenting the major hematologic characteristics of pernicious anemia in man. The evidence, presented by the experiments of Wolf, Weber, and Kröger, seems to be far distant from this ideal. Nevertheless, their report of the specific production of a classical reticulocyte crisis by administering liver extract to rabbits rendered anemic by typhoid toxin seemed worthy of repetition.<sup>2</sup>

#### METHOD

It is unfortunate that the article by Wolf, Weber, and Kröger fails to detail exact procedures in the preparation of the toxin. Their directions were followed, however, as closely as possible. A virulent typhoid strain was incubated at 37° C. for three weeks. The medium was composed of meat infusion

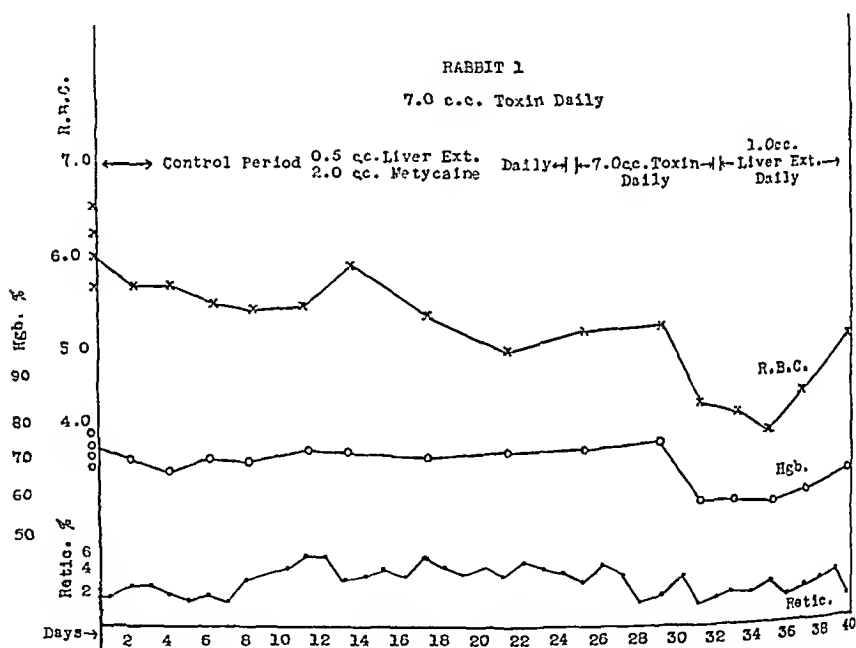


Fig. 1.

broth, Witte's peptone, and 0.2 per cent glucose, pH 8.2. Iron dialysate 5 per cent (Merek) was added in the ratio of 1:2, the resulting precipitate allowed to settle, and the supernatant liquid passed first through paper and finally through Berkefeld N filters. This filtrate was virtually protein free (sulfosalicylic acid and potassium ferrocyanide—acetic acid tests). Five liters of the filtrate were then concentrated at low temperature (25°-28° C.) vacuum distillation. The final product was clear, amber colored, and sterile.\*

Three white rabbits, weighing about 2 kg. each, were used. These animals were kept on a basic ration of oats with fresh vegetables added daily. All injections were given subcutaneously, local anesthetic followed by toxin at one site, liver preparation at another site.

\*We are indebted to Dr. John S. Lockwood and Dr. Harry M. Vars, of the Department of Research Surgery, for aid in the preparation of this material.

Concentrated liver extract\* and reticulogen\* were used as sources of hematopoietic liver principle for these experiments. The local anesthetic was metycaïne\* (1 per cent).

Erythrocyte, hemoglobin (Sahli), and hematocrit (Van Allen) values were determined every second day, leucocyte and platelet counts and blood film examination every fourth day. All counts were performed in duplicate, using certified pipettes and counting chamber. Reticulocyte counts (wet technique) were made daily. Several basal blood examinations were obtained for each rabbit before injections were started.

Rabbit 1 served as a control. Twenty three consecutive injections of 20 c.c. metycaïne and 0.5 c.c. liver extract were given. The observed changes in erythrocyte and hemoglobin values were not significant (Fig 1). Reticulocyte percentage increased irregularly to a maximum of 6.4 per cent on the

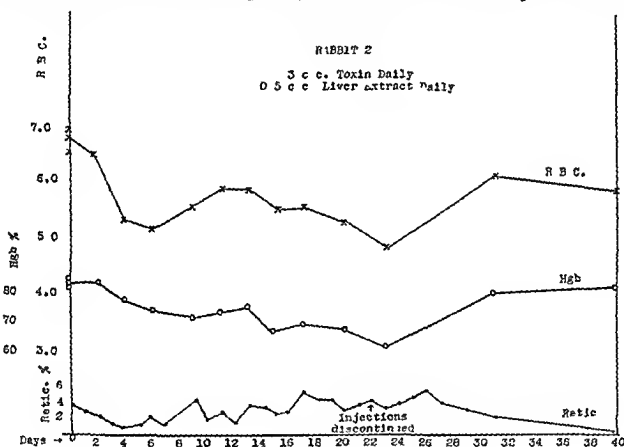


Fig 2

seventeenth day. A slight leucocytosis and thrombocytosis occurred. The animal remained entirely well, gained weight, and showed no dermal injury.

Using the same animal and beginning two days after completion of the liver injections (which totaled 11.5 c.c. of concentrated liver extract over a period of twenty three days), a course of toxin injections was instituted. The animal was given 20 c.c. metycaïne and 7.0 c.c. toxin (3.1 c.c. per kg. of body weight) daily for nine doses (Fig 1). There was a prompt, sharp fall in the red cell count and hemoglobin. Reticulocytes did not exceed 6 per cent. Leucocytes and platelets increased. The injections provoked local skin necrosis, induration, and occasional abscesses. The animal appeared listless and lost weight. Immediately after the toxin course, liver extract (10 c.c. daily) was again injected. Prompt improvement in red cell count and to a lesser degree in hemoglobin content was seen. There was no significant change in reticulocyte percentage.

\*Supplied by Eli Lilly & Co



Rabbit 2 was injected daily with 3.0 c.c. toxin (1.5 c.c. per kg. of body weight), 2.0 c.c. metycaine, and 0.5 c.c. concentrated liver extract (Fig. 2). A moderate, irregular anemia developed. Reticulocytes rose to a maximum of 6.1 per cent on the seventeenth day. Both erythrocyte and hemoglobin values improved soon after injections were discontinued on the twenty-second day.

Rabbit 3 received twenty-two injections of 5.0 c.c. toxin (2.2 c.c. per kg. body weight), 2.0 c.c. metycaine, and 0.25 c.c. reticulogen (Fig. 3). There was a progressive decline in red blood cell and hemoglobin values. The reticulocyte percentage began to fluctuate sharply on the sixth day; a peak value of 11.1 per cent was reached on the thirteenth day. Only after cessation of injections did the erythrocyte and hemoglobin values improve. The white cell and platelet counts increased soon after injections were begun. Both

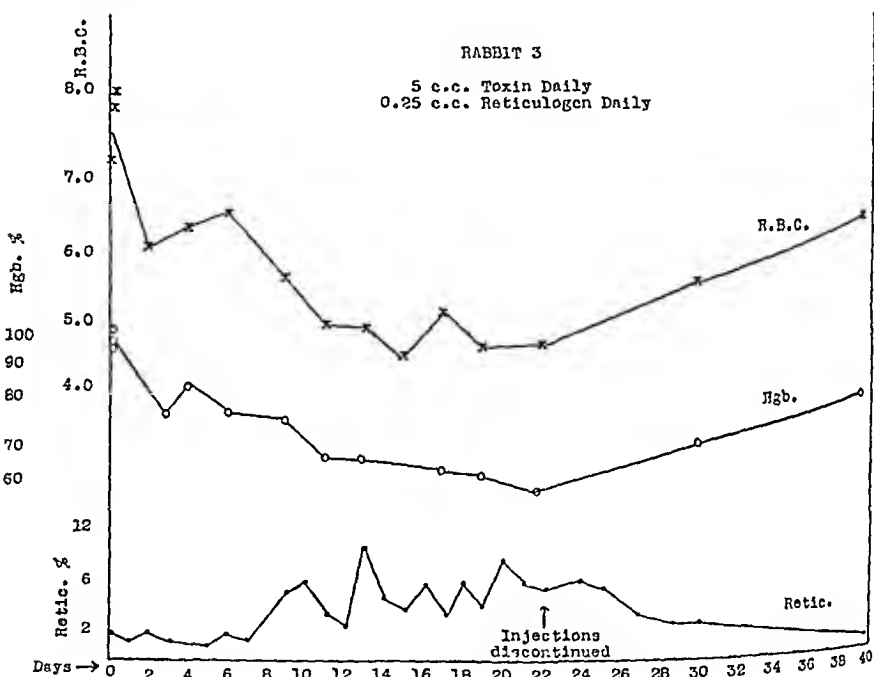


Fig. 3.

animals lost weight, seemed ill, and regularly developed skin necrosis with induration and occasional abscess formation wherever the toxin injection was made.

In none of the toxin-treated rabbits did any significant change in the leucocyte formula appear. There was a moderate increase in the relative and absolute numbers of immature neutrophils. Anisocytosis, poikilocytosis, and polychromatophilia were slightly more in evidence during anemic phases than during the control periods. Occasional normoblasts were noted. Hematocrit readings paralleled the erythrocyte-hemoglobin fluctuations.

#### CONCLUSIONS

It cannot be stated with certainty that the anemia produced in the toxin-treated rabbits was due entirely to the marrow-depressing action of typhoid toxin, as suggested by Wolf, Weber, and Kröger. The degree of local tissue

damage produced by the toxin seemed severe enough to be at least in part responsible for the illness and anemia. We were not able to reproduce the previously reported leucopenia and thrombopenia. On the contrary, our toxin treated animals, as well as the liver extract and metyrene control animal, developed leucocytosis and thrombocytosis.

With the appearance of anemia in the toxin treated animals there occurred a low grade reticulocytosis, irregular in onset and duration. The rabbit given 5.0 cc of toxin and 0.25 cc of reticulogen daily exhibited a moderate reticulocyte response but it was entirely unspecific in type. A well defined "peak" was not manifested. The control animal, which received at first only liver (and metyrene), showed practically comparable reticulocyte fluctuations.

Our observations do not confirm those reported by Wolf, Weber and Kroger. Typhoid toxin does not produce an anemia in rabbits which responds uniformly with a reticulocyte crisis to potent hematopoietic liver preparations. The method is unsuitable for assay of such preparations.

#### REFERENCES

- 1 Wolf, H. J., Weber, L., and Kroger, E. Die experimentelle Typhusanämie des Kaninchens als Test für die antiperniciöse Leberwirkung. *Med. Welt* 11: 1170, 1937.
- 2 Editorial, *Lancet* 2: 974, 1937.

## AN INEXPENSIVE SMALL CONSTANT TEMPERATURE BATH

MAURICE VAISBERG, M.D. PATCHOGLU, N.Y.

**I**N SMALL laboratories where extreme precision in temperature is not required, a constant temperature bath may be constructed at a very small cost.

1 The container is made by securing a two gallon oil tin, measuring 8 by 6 by 10 inches, free at any garage. For about \$1.00 the plumber can convert this to an open top double walled vessel, with all walls and bottom double. The air space between the walls varies from  $\frac{3}{4}$  to 1 inch. The conversion is made by soldering bright block tin around the original container.

2 The thermostatic control and heating element can be purchased at any fish and pet supply store. There is quite a choice of fish aquarium temperature regulators. An excellent one, costing about \$1.25, consists of thermostat, heating element, and temperature control all in one instrument. This is hung into the water from the edge of the container and is held securely in place by two adjustable metal bands. This is an exceptionally accurate instrument for one costing so little. In a continuous five day test it kept two gallons of water at a constant temperature of 38° C., with but one half degree total variation either way.

3 To stir the water in the bath, one may use either one of the two following:

A. At the large chain stores one can obtain for about \$1.19 a small electric egg beater made by the Chicago Electric Mfg. Co., and called "handywhip". It consists of a mixing bowl, a sturdy induction motor, and a detachable beater.

or agitator. It should be used in series with a 30 or 40 watt lamp. This prevents overheating and also slows the agitator down to the proper speed. It is a very simple matter to rig up a small series-parallel board of three outlets on a little piece of wood.

B. A fish bowl aerator pump may be used. These sturdy little induction motor pumps cost about \$15.00 but can be used for a large variety of aerating and stirring procedures. They have been known to run continuously for three years.

The apparatus is set up in the following manner. A sizable ringstand and base are used. The container is set on the base with its long axis at right angles to the long axis of the base. Water of about the desired temperature is added to near the top, and the thermostat-heater is inserted over the side and adjusted by a little experimentation. A clamp attached to the ringstand supports a thermometer in the water. Another clamp supports the stirring motor and agitator; or, if the pump is used, a glass tube extends down to the bottom. Other clamps support the flasks containing the solutions being tested and incubated.

The induction motor of the egg beater will no doubt please a large number of laboratory workers who will find an infinite variety of uses for it. One can detach the agitator and put on a small propeller and use it in the top of an incubator to secure forced draft ventilation. Here it should be used in series with a 40 watt lamp. It is also a most excellent motor to stir small quantities of inflammable material and solutions as ether and acetone. Since it is an induction motor, there is no danger of sparks; there are no brush replacements, and there is no radio interference. In this laboratory it was used as an inexpensive motor to run a homemade kymograph. On a test run, the motor ran continuously (in series) for three days, without the slightest evidence of overheating.

The total cost of the set-up is as follows:

Container	\$1.00
Motor	\$1.19
Thermostat heater	\$1.25
Incidentals	\$0.56
Total	\$4.00

(Prices are subject to some variation in different localities.)

## THE REFINING OF ANTISERA\*

### II. IMPROVEMENTS IN THE PREPARATION OF REFINED AND CONCENTRATED PNEUMOCOCCUS AND MENINGOCOCCUS ANTIBACTERIAL SERA

EUGENE CARDONE AND K. GEORGE FALK, NEW YORK, N. Y.

#### INTRODUCTION

A METHOD of refining and concentrating antisera was described four years ago.<sup>1</sup> Since then a number of changes have been introduced as continued experience with the procedures warranted their incorporation. These changes will be described here. The alphabetical sequence given in the former paper in the description of the successive procedures will be used, the paragraphs designated with a definite letter referring to the paragraph with the same letter in the former paper. The changes apply equally to the refining of pneumococcus and of meningococcus antisera.

#### EXPERIMENTAL PROCEDURE

a. *Preparation of Serum.* Immediately after separation from the clot, 0.3 per cent phenol, in the form of a solution containing three parts of phenol and five parts of ether, and 0.005 per cent merthiolate are added.

b. *Dialysis of Serum.* Dialysis is carried out in a cold room at 7° to 10° C., the running tap water in the tank being at the same temperature. Dialysis is allowed to proceed for six days against a comparatively rapid flow of water. No additional phenol is added during dialysis.

c. *"Acid Protein" Precipitation.* Immediately after dialysis solid sodium chloride is added to the cold serum to N/20, and the pH adjusted to 5.1 with normal acetic acid ( $\gamma$ -dinitrophenol as indicator). This is done in the late afternoon. The material is allowed to stand at 7° to 10° C. overnight. The removal of "acid protein" by filtration through paper pulp and "super cel" is carried out as before.

d. *Preliminary Tests for Antibacterial Substance Preparation.* Four 50 ml. portions are adjusted with N/sodium hydroxide to the pH values 5.9, 6.1, 6.8, and 6.8. The first three are diluted with four volumes of distilled water while the last (pH 6.8) is diluted with six volumes. The precipitates are allowed to settle in the cold, and are then centrifuged and dissolved in 10 ml. portions of 1 per cent sodium chloride solution. Supernatants and dissolved precipitates are tested by the precipitin reaction. The precipitate portions show potency and recovery of material; the supernatants, the optimum hydrogen ion concentration for bulk precipitation not incurring loss.

\*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.

Received for publication, April 22, 1938

e. *Precipitation of Antibacterial Substance.* No preservative is added to the distilled water used in the precipitation. The precipitated antibacterial substance is allowed to settle overnight in the cold, and finally centrifuged.

f. *Solution of Antibacterial Substance.* The precipitate is dissolved in the smallest practicable volume of a solution of 2 per cent sodium chloride and 0.5 per cent phenol (about one-twentieth of the original volume as a rule). The final antibacterial solution is to contain 1 per cent sodium chloride and 0.31 per cent phenol. The above solution is, therefore, diluted with distilled water containing the calculated additional amounts of sodium chloride and phenol to give the required final volume as determined from the preliminary tests and the solid content desired (usually one-seventh to one-fifteenth of the original volume). The solution is adjusted to pH 6.8 with N/sodium hydroxide if the precipitating pH value was less than this, and 0.005 per cent merthiolate is added. The material is kept in the cold. After twenty-four hours, the preparation is centrifuged to eliminate the coarser particles of separated fibrin material. This procedure facilitates the subsequent filtration through paper pulp and "super cel" (Buchner funnel) and the final passing through a small (6 inches) Berkefeld V filter.

g. *Alternative Method of Precipitation of Antibacterial Substance.* If an excessive amount of antibacterial substance is found in the supernatants of all the test portions, the material is not precipitated with ammonium sulfate to 50 per cent saturation as described in the previous paper. Instead, precipitation tests are run on portions of serum at the same hydrogen ion concentrations as before, but with the addition of six and eight volumes of water in place of four. This procedure almost invariably causes complete precipitation of antibacterial material with antibacterial-free supernatants. The bulk of the serum is adjusted to the determined hydrogen ion concentration, and the volume of distilled water needed for complete precipitation is added.

#### NOTES ON THE EXPERIMENTAL PROCEDURE

A. The method of purification and concentration described is applicable mainly to antisera. If the procedure is used with antiplasmas, several difficulties arise. Upon dialysis the intake of water is very large, frequently equal to the original volume of the plasma. This dilution makes the preparation more difficult to handle. Furthermore, the fibrin present adds to the difficulties in the filtration through fine-pored pulp and super cel. Even if these complications are surmounted satisfactorily, so much fibrin is found in the concentrated material that final filtration and Berkefelding is accompanied by appreciable loss.

B. As stated in the former paper, it is believed that dialysis of serum not only eliminates most of the salts but also causes changes in the structure of the proteins. Furthermore, when phenol is added during dialysis, other changes may occur, such as the binding of hematin material to the protein aggregate particle. This combination is not readily broken down in the subsequent purification, and the final product may have more color than if additional phenol had not been added during dialysis. It has been determined definitely that, even if the phenol which is removed in the dialysis is not replaced, sterility of the product is maintained. The original serum must, of course, be sterile.

C. Formerly, all types other than I and II were allowed to remain at pH 5.1 for only five hours. Now, all sera remain at this pH overnight to allow complete precipitation of "acid protein." This technique results in no loss by inactivation.

D. The preliminary tests to determine the best hydrogen ion concentration for antibacterial substance precipitation are still very essential. However, the pH 6.3 sample is not prepared any more. A pH 6.8 sample diluted with six volumes of water has been substituted for it. If the supernatants of all these tests show no antibacterial protein, the pH 6.8 value is preferred. Appreciably less fibrin is carried down with the precipitating protein at this pH. The final preparation also filters more readily and has a better appearance. A serum, which shows loss when precipitated at any volume (within workable limits) at pH 6.8, is encountered at times. For this reason, the pH 5.9 and 6.1 samples are still prepared.

E. The addition of 0.2 per cent phenol to the precipitating water has been given up as unnecessary. Also, recent work has shown that part of the phenol is adsorbed by the precipitated protein.<sup>2</sup> The material is kept at refrigerator temperature throughout.

F. Preparations which are precipitated at pH 5.7, 5.9, or 6.1, gave clear solutions when dissolved. However, when the pH's of these solutions are changed to 6.8-7.0, a suspension or cloud appears, probably caused by fibrin which had been adsorbed and which is set free by the change in pH. This suspension or cloud becomes more pronounced in the cold.

G. The addition of 0.35 per cent phenol and 0.005 per cent merthiolate, in place of 0.5 per cent phenol, is used to ensure sterility.<sup>3</sup>

#### DISCUSSION

The changes in the method of concentrating and refining the antibacterial material of sera which are described have given more satisfactory products and better recoveries or yields with a considerable number of preparations. The potencies and clinical efficacies of these preparations do not come within the scope of this paper, except for the fact that the method would necessarily be abandoned if certain standards were not attained.

The use of phenol during the various procedures has been reduced as far as possible. Apparently, changes in the proteins are either induced or increased by phenol, changes which may interfere with the purification or produce other untoward effects. Furthermore, as shown elsewhere,<sup>2</sup> phenol is adsorbed by precipitated proteins in considerable quantities. This adsorbed phenol must be reckoned with in the final products when phenol is added to a definite concentration to ensure sterility. It cannot be too strongly emphasized that phenol estimations should be carried out sufficiently frequently, not only on the final products but also at various stages of the manipulations involved in the refining and concentrating procedures, to control the actual quantities present at all times.

Very little of a definite nature can be added at the present time to the theory of the molecular changes involved in the treatments described in these procedures. That aggregation of molecular units is involved at certain stages

and break down at other stages appear unquestioned, although it is obviously difficult to obtain quantitative evidence of their extent.<sup>4-6</sup>

Reference to the former paper may suffice instead of repeating the discussion already presented of various factors of interest.

#### SUMMARY

Improvements in the procedure used in this laboratory for the purification and concentration of antibacterial sera, particularly those of pneumonia and meningitis, are presented.

Thanks are due the members of the Pneumonia Division of this laboratory for their constant cooperation, and especially Miss Katherine Blount for carrying out the large number of precipitin tests.

#### REFERENCES

1. Klein, A. J., Greenwald, C. K., and Falk, K. G.: The Refining of Antiserums, *J. LAB. & CLIN. MED.* 19: 1324, 1934.
2. Cardone, E., and Mazzarella, R.: The Adsorption of Phenol by Protein in Preparation of Certain Biologic Products, *J. LAB. & CLIN. MED.* 24: 137, 1938.
3. Falk, C. R., and Aplington, S. P.: Studies on the Bactericidal Action of Phenol and Merthiolate Used Alone and in Mixtures, *Am. J. Hyg.* 24: 285, 1936.
4. Svedberg, T.: The pH-Stability Regions of the Proteins, *Tr. Faraday Soc.* 26: 749, 1930.
5. Idem: Ultracentrifugal Dispersion Determinations of Protein Solutions, *Kolloid-Ztschr.* 51: 10, 1930.
6. Reiner, H. K., and Reiner, L.: Fractional Precipitation of Serum Globulin at Different Hydrogen Ion Activities. Experiments With Globulin Obtained From Normal and Immune (Antipneumococcus) Horse Serum, *J. Biol. Chem.* 95: 345, 1932.

## THE USE OF NONCOOKED, NONSTERILIZED COCONUT MILK AS AN ADDITIONAL NUTRIENT SUBSTANCE IN CULTURE MEDIA\*

LEE BLAUVELT, M.T., ASHEVILLE, N. C.

IT HAS long been known and appreciated that overheating of culture media impairs their growth qualities. It is even true that in some cases valuable characteristics of the organism are not exhibited in these overheated media.

It is logical to believe, therefore, that the growth quality would be enhanced and the characteristics of organism more accurately demonstrated if an uncooked nutrient were added to cooked and sterilized media.

This fact has also been realized and met by the use of a number of agents, among which the best known are probably blood, ascitic fluid, hydrocele fluid, pleural fluid, and potato. All of these have definite uses for which they cannot be easily replaced. But their use outside these specific instances has been limited. The four former substances cannot always be obtained, and in the case of the potato, special care must be employed to maintain sterility during processing.

In 1891, Van Slyke referred to the use of coconut milk as a bacteriologic culture medium. In 1897, Erwin F. Smith used coconut meat as a medium for yeast and molds. And in 1920, B. A. Linden utilized coconut juice or milk as

\*From the Biltmore Hospital, Asheville.

Received for publication, April 25, 1938.

culture and by fractional sterilization made an agar medium. But I have been unable to find reference to the use of coconut milk in the unsterilized state as an uncooked nutrient substance which may be added to general culture media to enhance their nutrient properties.

In the following experiments, the juice of fresh, ripe coconuts was used, the ripe fruit was used rather than the green, because it is more readily obtainable. The coconuts were obtained as fresh as possible. The eyes were soaked in alcohol and then burned. A large aspirating needle was then inserted, stylet in place, in the softer eye (stylet withdrawn and sterile syringe attached), and the milk withdrawn. (The average coconut was found to contain 100 to 125 cc of slightly turbid fluid.) Smears were made from the centrifuged sediment of a portion of the fluid to determine the presence of possible organisms. The fluid was then added in the proportion of 10 to 25 per cent to broth or agar medium. The medium was then adjusted to the pH recommended for the organism under consideration, and the tubes inoculated in the manner required by the specific organism. Toward the end of the coconut season and before the fresh fruit comes to market, it was found well to incubate the milk overnight in a sterile corked container, to insure it against the presence of organisms.

The organisms used in this experiment were *Staphylococcus aureus*, *Streptococcus viridans*, *B. fecalis alcaligenes*, pneumococcus, gonococcus, meningococcus, *B. welchii*, and tubercle bacilli. In each instance, unless otherwise stated, the medium to which the coconut milk was added was either plain nutrient broth, plain agar, or both.

I In the case of the *Staphylococcus aureus*, 2 per cent glucose agar and plain nutrient agar were used in comparing media with the coconut milk enriched media. After twelve hours of incubation, the colonies on the coconut milk media were half as large again as those on the glucose medium and slightly more numerous. The growth on the enriched agar was twice that on the plain agar.

II In the case of the *Streptococcus viridans*, blood agar was used as a comparing medium. After twelve hours incubation, growth was nearly twice as abundant on the blood agar, but there was good growth on the coconut milk medium. This being true, the organism was replanted on 2 per cent glucose and again on the coconut milk medium. In this instance, the growth was one and one half times greater on the latter. Smears were made from both these last cultures, and the organisms grown on glucose agar at twelve hours showed very short chains and often no chains at all, while those on the coconut milk agar showed longer and more characteristic chain formation. Although in this case the coconut milk medium is not superior to the blood agar, it was found to be superior to the glucose agar. This fact can be of value to office and small institutional laboratories where suitable blood is not always available. Comparative cultures were also made on enriched and plain nutrient broth. Growth was doubled in the enriched broth.

III In the case of *B. fecalis alcaligenes*, plain agar and nutrient broth were used as comparing media. Growth after twelve hours was twice as great and the colonies more luxuriant on the coconut milk agar than on the plain. Broth culture was more turbid in the case of the coconut milk broth.

IV Blood agar was used as a comparing medium for the study of the



pneumococcus. Again as in the *Streptococcus viridans*, the growth was only about half as great on the coconut milk agar as on the blood agar. However, smears from both cultures showed definite short chain and capsule formation. Again as in the case of the *Streptococcus viridans*, further comparison was made between plain agar and the coconut milk enriched agar. The colonies were larger and the growth nearly twice as great on the coconut agar. Smears from these two cultures showed chain and capsule formation as before on the coconut agar, and only a rare chain on the plain agar.

Swartz medium and plain agar were used as comparison for the study of the gonococcus. The reading in this case was made in seven days. Moisture was maintained and the oxygen tension kept as nearly 20 per cent as possible. Growth on the Swartz medium was approximately one-fourth greater than on the enriched plain agar, but there was quite a visible growth on the latter. This fact should again be of value to small laboratories where ascitic, pleuritic or hydrocele fluid is not always available. No growth was obtained on the plain agar culture.

Blood serum medium and plain agar were used for comparison for the meningococcus. In this instance, the growth appeared more rapidly on the coconut milk agar but was less luxuriant after growth was established. Growth was absent to scant on the agar cultures.

Plain broth and plain agar were used for comparison for the *B. welchii* concentrated beef broth. In the concentrated broth and the coconut broth growth was almost equal and in each instance luxuriant. Growth was much less luxuriant on plain agar and in plain beef broth.

Potato medium and plain agar were used for comparison in the work on tubercle bacilli. The coconut medium did not shorten the period of lag, and growth was about half as heavy on it as on the potato medium. There was no growth on the plain agar slants. In this instance, the medium to which the coconut milk was added was an egg yolk glycerin agar.

An analysis of coconut milk, reported by E. M. Caray in the *Philippine Agriculturist*, was obtained through the courtesy of the bacteriological section of the U. S. Food Division and is as follows:

Specific gravity at 15.5	1.023
Water at 60° C.	0.96 per cent
Ash	0.9 per cent
Acid constituents	48.0 per cent
Glucose	0.5 per cent
Cane sugars	2.6 per cent

The sugars present were found to be sucrose, fructose, and glucose; the per cent was given only for those mentioned in the above table.

The vitamin content of coconut milk made by J. H. Axmayer, also obtained through the U. S. Food Division, states that "Vitamin B is present in small amounts, while factor G (B<sub>2</sub>) is present in appreciable quantities. Vitamins A and B have been reported in the meat."

#### SUMMARY

Sterile coconut milk is easily obtained by aseptically aspirating ripe fresh coconuts. The addition of this nonecooked, nonsterilized nutrient agent nearly doubled the cultural qualities of ordinary nutrient broth and agar in the case

of all organisms tested, and was found to be superior to the usual medium used in the case of *Staphylococcus aureus*, *B. fecalis alcaligenes*, and *B. welchii*. In the case of the meningococcus, it was found to be equally as good as the medium usually used. In the cases of the *Streptococcus viridans*, pneumococcus, gonococcus, and tubercle bacilli, it was found to be inferior to the preferred selected media.

## REFERENCES

- 1 Cary, Elms The Chemical Composition of Copra Meal With Special Reference to Its Carbohydrates, The Philippine Agriculturist 1921 p 51
- 2 Behre, A The Composition of Coconut Milk, Cited in Chemical Abstracts 1 804, 1907
- 3 Van Slyke, L. L. Analysis of Milk of Ripe and Unripe Coconuts, Am Chem J 13 130, 1927
- 4 Matthews, C. G. Notes on the Liquid From Ripe Coconuts Reviewed in Chemical Abstracts 19 1002, 1925

## PREPARATION OF COLLOIDAL GOLD SOLUTION\*

R. D. SCOTT, B.S., AND GOLDIE DE LONG COLUMBUS OHIO

ON SEARCHING the literature it appears the first recorded preparation of colloidal gold solution, or gold sol, was made by Faraday<sup>1</sup> in 1857. His method was the reduction of a solution of gold chloride by phosphorus dissolved in ether.

Biedig<sup>2</sup> in 1897 prepared gold sol, passing an arc between gold electrodes under water.

Zsigmondy<sup>3</sup> in 1898 presented the method of reducing gold chloride in solution made alkaline with potassium carbonate using formaldehyde as a reducing agent. He also made the important observation that flocculation of gold particles with an electrolyte such as sodium chloride is prevented by definite protein concentrations, specific for each protein.

To Lange<sup>4</sup> is due credit for adapting this phenomenon to the examination of spinal fluids. He observed in 1912 that in suitable saline concentration those with syphilitic involvement cause flocculation of gold while normal spinal fluids do not. He proposed the scale of dilutions, color ratings, and form of reporting now used in distinguishing the several types of syphilitic affections of the central nervous system.

Various modifications of Zsigmondy's preparation technique have been proposed by numerous investigators, their number attesting to the difficulty of preparing a satisfactory solution. Those most in use are of three general types: (1) the Eike<sup>5</sup> modification (1913), using glucose instead of formaldehyde, (2) the Miller<sup>6</sup> modification (1915), using oxalic acid together with formaldehyde, and (3) the Mellaub<sup>7</sup> a new method (1923), using potassium oxalate without formaldehyde.

The glucose method yields beautiful preparations which, however, do not seem so well adapted for clinical use as those prepared by the Miller procedure, since in our experience they are less sensitive. While we have not tried

\*From the Ohio Health Department Laboratories Columbus  
Received for publication May 16 1938

out the oxalate procedure of Mellanby, it does not appear suitable for the preparation of large quantities.

A satisfactory colloidal gold solution may be defined as one which gives typical reactions with pathologic spinal fluids and no reaction with normal fluids, under the test conditions.

Satisfactory preparations will have the following characteristics:

The color by transmitted light is red, variously described as rose red, salmon red, or cherry red, with no trace of blue tint. By reflected light the color is brown, clear or with only slight turbidity.

The reaction lies between pH 6 and 7. Five volumes are precipitated by 1.7 volume of 1 per cent sodium chloride, largely within an hour; completely overnight.

Usually, but not always, such solutions will be found satisfactory for clinical use.

Numerous factors influence the preparation of gold sol. There is lack of agreement both as to their relative importance and whether a given factor is favorable or the contrary. Thus, some observers believe that the distilled water used should be of the highest purity obtainable; others contend that traces of certain impurities are helpful, for example, those derived from rubber connections.

Some maintain that sunlight is necessary; others hold that its effect is not material. Some prescribe the addition of the several reagents separately and at definite temperature stages; some add them together.

The temperature of reduction is considered critical, but temperatures of 85°, 90°, 95°, and 100° C. are variously recommended.

Some workers use involved methods of adjusting the final reaction to neutrality; others consider it unnecessary or impractical to attempt any correction. Some prefer rapid heating, some slow. To date, over a period of four years, we have prepared 320 liters of satisfactory colloidal gold and some which were unsatisfactory. We have experienced considerable difficulties, but more in the past than at present.

We employ the Miller modification in so far as using both oxalic acid and formaldehyde as reducing agents.

The procedure of Miller and his co-workers is outlined: Heat 1000 c.c. of distilled water over a burner. At 60° C. add 10 c.c. of 1 per cent gold chloride and 7 c.c. of 2 per cent potassium carbonate. At 80° C. add 10 drops of 1 per cent oxalic acid. Remove from flame at 90° C. and add 5 c.c. of 1 per cent formaldehyde (40 per cent solution diluted 1 in 40), or enough to produce a pink color.

We found that double distilled water seemed to work better than triple and following the installation of a new, very efficient still we were able to use single distilled water, letting the distillate come over warm and receiving it in a pyrex bottle.

It was found unnecessary to adhere to the prescribed temperatures; the gold chloride, potassium carbonate, and oxalic acid may be added successively, with stirring, at temperatures between 50° and 60° C. Heating is then continued to boiling instead of 90°, the beaker is then removed from the flame and the formaldehyde is added. We believe that the reduction proceeds more

rapidly in good daylight and that it is promoted by the presence of a previously formed film of gold on the beaker wall, also by sufficient stirring

In our opinion the most important factor which determines whether the solution is clear and satisfactory for the purpose of the test, or turns turbid and unusable, is the technique of the reduction with formaldehyde

It was observed that the amount added, the time allowed for reaction, and the temperature are all of significance. Accordingly, we gradually evolved the procedure of adding a definite volume and depend on the time and temperature factors for completion of the reaction

Our procedure, which we have employed for the past four months, by means of which over 100 liters of uniformly satisfactory sol have been prepared, is to add at once 30 cc of the formaldehyde solution, then to stir continuously. A pink color soon develops gradually deepens, then suddenly flashes to the desired light cherry red color. This final color change usually develops as the temperature drops to from 85° to 80° C.

Immediately after preparation, the solution is transferred to pyrex bottles and is stored in the dark, under refrigeration. The beaker used is partially, but not completely, freed from deposited gold film by wiping with a clean towel, and after rinsing with distilled water is again ready for use. The strength, as well as the purity of the reagents used, must be considered. We have noted that while different lots of gold chloride oxalic acid, and formaldehyde have not deviated materially from their theoretical contents, three brands of C P grade so called anhydrous potassium carbonate ranged in alkaline strength from 82 to 89 per cent of theory. Thus varying strengths of this reagent may also tend to explain the difficulties of preparation.

We have found through experience—not by calculation—that using potassium carbonate 83 per cent anhydrous strength, the optimum volume in relation to the other reagents used is 7.8 cc of 2 per cent solution.

It has been our experience, using the oxalic acid formaldehyde method of reduction, that if the finished solution is clear, or nearly so, and is free from blue tint, it will almost invariably be found satisfactory in actual use.

We make no adjustment in reaction after preparation and determine whether a given solution is suitable for use by its behavior with known normal and paretic spinal fluids. If slightly insensitive then in the next lot prepared we add 3.2 cc formaldehyde solution instead of 3.0 cc, if slightly oversensitive, we add 2.8 cc in the next preparation.

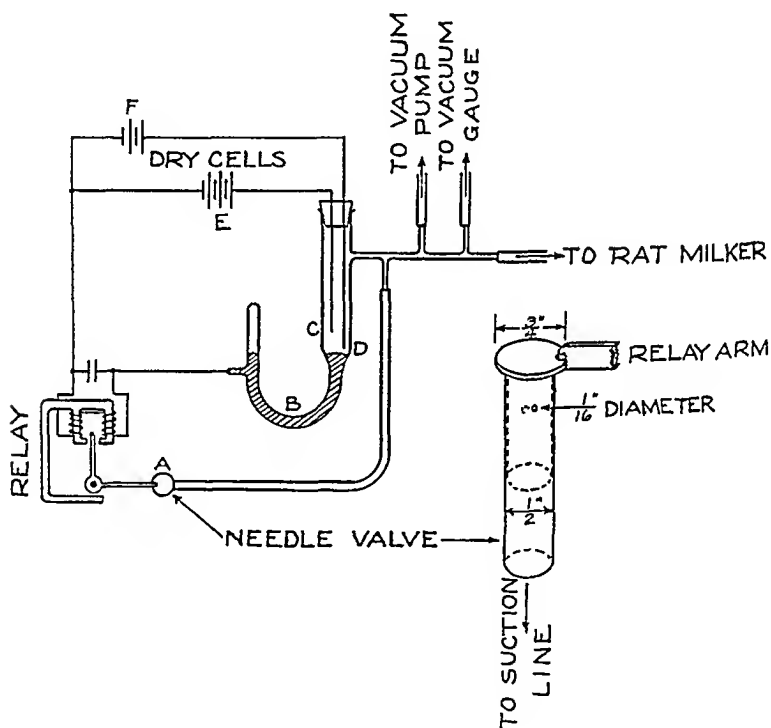
#### REFERENCES

1. Poschl, Victor. *The Chemistry of Colloids*. London, 1910. Charles Griffin & Co., pp 52, 53.
2. Bredig, G., and Muller von Berneck, R. *Ueber Anorganische fermente*, *Ztschr f physiol Chem* 31: 258, 1899.
3. Zsigmondy, Richard. *Die hochrote Goldlosung als Reagens auf Colloids*, *Ztschr f anal Chem* 40: 697, 1901.
4. Lange, Carl. *Ueber die Ausflockung von Goldsol durch Liquor cerebrospinalis*. *Klin Wchnschr* 49: 897, 1912.
5. Ficke, Hans. *Die Goldreaktion im Liquor cerebrospinalis*, *Munchen med Wchnschr* 60: 2713, 1913.
6. Miller, Sydney R., Brush, Nathaniel D., Hammers, James S., and Felton, Lloyd D. *A Further Study of the Diagnostic Value of the Colloidal Gold Reaction*, *Bull Johns Hopkins Hosp* 26: 391, 1915.
7. Mellanby, John, and Ansell Davies, Thomas. *The Precipitation of Colloidal Gold by Cerebro Spinal Fluid*, *Brit J Exper Path* 4: 132, 1923.

# A MODIFIED APPARATUS FOR MILKING SMALL LABORATORY ANIMALS\*

ARTHUR J. MUELLER, B.S., EVANSVILLE, IND.

IN 1937, Cox and Mueller<sup>1</sup> described an apparatus suitable for milking small laboratory animals. The essential part of this report was the perfection of conditions necessary to obtain milk from rats. As an added convenience, an automatic device for obtaining pulsations in negative pressure (through the clamping action of a magnetic switch) was described. Temple and Kon<sup>2</sup>



confirmed the original findings and obtained pulsations in negative pressure through the operation of a piston.

The present note deals with a simplification of the early apparatus.<sup>1</sup> The magnetic switch (which operated with some uncertainty and much noise) has been replaced by a needle valve operated by the movable arm of an ordinary polarized telegraph relay. The entire apparatus (as indicated in Fig. 1) is readily constructed, and operates quietly and efficiently.

When suction is applied, the mercury rises in the U-tube, *B*, and completes the circuit at electrode, *D*. The current from the two dry cells, *F*, is insufficient

\*From the Mead Johnson Research Laboratories.  
Received for publication, May 6, 1938.

to throw the relay and break the suction. When the mercury rises to electrode *C*, the effect of the three cells, *E*, throws the arm of the relay and opens the needle valve *A*, admitting air. When the contact at *C* is broken, the residual current (from *F*) is adequate to hold the needle valve (relay) in the "open" position. When, however, contact is broken at *D*, the arm of the relay, animated by the permanent magnet of the relay, closes the needle valve and the cycle begins again. This system of balanced electrical currents gives the lag necessary in effective milking.

The needle valve is essentially two machined tubes with small holes in close approximation. The notched head which fits into the arm of the relay, is attached to the inner tube, while the outer tube (connected to the suction line) is held firmly in place and is machined to fit tightly against the under side of the notched head. The two small holes are adjusted to open or close with the movement of the arm of the relay.

#### REFERENCES

- 1 Cox, Warren M. Jr., and Mueller, Arthur J. The Composition of Milk from Stock Rats and an Apparatus for Milking Small Laboratory Animals. *J. Nutrition* 13: 249, 1937.
- 2 Temple, P. L., and Kon, S. K. A Simple Apparatus for Milking Small Laboratory Animals. *Biochem. J.* 31: 2197, 1937.

## A METHOD FOR THE DETERMINATION OF BROMINE IN A PROTEIN FREE FILTRATE\*

HYLEN L. WIKOFF, PH.D., ELOISE BAMP, M.A., AND MARCEL BRANDT, M.A.  
COLUMBUS, OHIO

THE recent interest in bromine metabolism and medication has brought an imperative need for an accurate and simple method for determining bromine in blood. The existing methods for the determination of bromides are too complicated and time consuming for routine use in the hospital. The first step in nearly all of these procedures requires preliminary destruction of organic material. This is accomplished either by drying the sample and igniting it with an alkali<sup>1</sup> under carefully controlled temperature conditions, or by the wet oxidation<sup>2</sup> of the sample in acid. Whether the organic material is destroyed by the wet or dry method, the bromine is usually separated from the other halides by selective oxidation in an acid medium followed by distillation or aeration of the bromine or its oxidation products into a suitable absorption system.<sup>3a, 3d, 3f, 3j, 3k, 2a, 2c</sup> The amount of bromine absorbed may be measured by the iodine liberated from added potassium iodide. The iodine may be determined either volumetrically or colorimetrically.

Another colorimetric procedure<sup>3e</sup> depends upon the production of a pink color (eosin) when an aqueous extract of the ashed sample, buffered with an acetic acid sodium acetate solution, is treated with chloramine T after addition of

\*From the Department of Physiological Chemistry, College of Medicine, Ohio State University.

a drop of alkaline fluorescein solution. Color comparisons are made with test tube standards containing varying concentrations of bromine similarly treated.

In still another colorimetric procedure the bromide ions in an aqueous extract of the ashed filtrate are oxidized to bromine in the presence of decolorized fuchsin.<sup>1g, 1h, 2e</sup> This procedure gives rise to the development of a lavender or pink-colored compound which can be isolated on Fuller's earth and then redissolved in chloroform for use in a colorimeter (Denigès and Chelle reaction<sup>3</sup>).

Malamud<sup>4</sup> developed a colorimetric method for the determination of bromides based on the production of a brown color following the addition of a solution of gold chloride to a protein-free blood filtrate. This procedure greatly shortens the time required for a blood bromide determination and gives consistent results with blood drawn from patients receiving bromine medication (extremely high blood bromide content). However, the bloods of normal persons and patients with low blood bromide content, as determined by the method to be described, do not contain enough bromine to produce a satisfactory color with gold chloride.

Using a potentiometric method with a silver-silver bromide electrode, Hastings and van Dyke<sup>5</sup> titrated a protein-free filtrate with silver nitrate and obtained a curve from which the amount of each halide present could be determined. This method is cumbersome and requires the use of fairly expensive apparatus with which the ordinary laboratory technician is not usually familiar.

The method to be described in this paper consists of a modified Denigès and Chelle reaction applied directly to a protein-free filtrate. The oxidation of organic material is omitted since our experiments to be described later show this step to be unnecessary. A modification of the colorimetric procedure as originally described is the substitution of acetone for chloroform as a solvent for bromofuchsin derivative. The relative solubilities of the halogenated fuchsin derivatives are the same for acetone as for chloroform. However, the colors developed in an acetone solution are far better for colorimetric work than those developed in chloroform solution, and since acetone and water are miscible, there is less tendency for an acetone solution to be cloudy.

#### EXPERIMENTAL PROCEDURE

*Reagents.*—(1) Fuchsin reagent. A solution prepared by dissolving 0.65 gm. basic fuchsin in 500 c.c. warm water is cooled and treated with 5.0 c.c. concentrated hydrochloric acid. Five cubic centimeters saturated sodium bisulfite solution are then added, and the solution allowed to stand until decolorized (overnight). Such a solution is stable for three or four months at room temperature.

The sensitivity of this reagent to bromine may be increased by aerating the excess sulfur dioxide from the solution. However, the resulting solution is extremely unstable at room temperature and becomes colored within twelve hours, but if kept in a refrigerator this solution remains colorless for nearly a year. Nevertheless, the unaerated solution is much more practical for routine analyses.

(2) Bromine standards. (a) Stock solution—0.001 gm. bromine per c.c. prepared by dissolving 1.49 gm. potassium bromide in a liter of distilled water.

(b) Working standard—0.000001 gm (1 gamma) per c.c. prepared daily by making appropriate dilutions of the stock solution

- (3) Concentrated sulfuric acid
- (4) Potassium persulfate
- (5) Saturated sodium bisulfite
- (6) Fuller's earth
- (7) Acetone

*Procedure*—A small amount of persulfuric acid is formed by adding 3 or 4 drops of concentrated sulfuric acid to 30 or 40 mg potassium persulfate in a clean dry test tube and allowing them to react for three or four minutes. Ten cubic centimeters of a protein free blood filtrate prepared according to the usual procedure of Folin and Wu are then added followed by 1 c.c. fuchsin reagent. Within a few minutes after mixing the reagents by inverting the test tube, the solution becomes violet colored, due to the reaction of the bromine (formed by the oxidation of the bromide with persulfuric acid) and the fuchsin reagent. After this solution stands for a few minutes, the color changes to pink, this indicates that the oxidation of the bromine has been completed. The action of the persulfuric acid is then stopped by adding a drop of saturated sodium bisulfite solution. This treatment restores the violet color of the solution.

The halogenated compounds of fuchsin are absorbed on about 25 gm Fuller's earth mixed well with the solution. After centrifugation, the supernatant liquid is discarded and the tube allowed to drain for two or three minutes. The bromofuchsin compound is then separated from the Fuller's earth by shaking well with 5 c.c. of acetone. After a second centrifugation, the pink colored solution is decanted and compared in a colorimeter with a standard containing 10 gammas bromide solution treated in the same way.

When more than 5 mg bromine per 100 c.c. are present (bloods from patients receiving bromide medication), the colored compound is not completely absorbed on the Fuller's earth. However, acetone (3 c.c.) added directly to the colored solution after the addition of the saturated bisulfite solution, dissolves the colloidal bromoderivative producing a color suitable for comparison with a standard treated in the same way.

BLOOD BROMINE CONTENT OF 10 STUDENTS

Student No	Br in mg per 100 c.c. whole blood
1	0.71
2	1.57
3	0.92
4	0.65
5	0.64
6	0.64
7	1.12
8	0.89
9	1.00
10	0.12

#### DISCUSSION

The use of acetone as a solvent for the bromofuchsin compound produces a solution having a much deeper and more intense color than when chloroform is used. Small differences in bromine concentration are much easier to detect



colorimetrically. Furthermore, since acetone is soluble in water, temporary emulsions which form between chloroform and water are avoided. The relative solubilities of the halogenated fuchsin compounds are the same for chloroform as for acetone, the iodo and the bromo derivatives being soluble and the chloro derivative insoluble in acetone.

The mere presence of bromine in a protein-free blood filtrate does not preclude the existence of bromine in the protein material precipitated when preparing the filtrate. Therefore, this material was dried and examined separately for bromine. Three different procedures for the destruction of the organic material were as follows: (1) ashing the dried precipitated protein material at 450-500° C. in the presence of five times its weight of sodium carbonate, (2) wet oxidation with a chromic sulfuric acid mixture, and (3) wet oxidation with a chromic sulfuric phosphoric acid mixture. Solutions prepared in the first case by dissolving the ash in water, in the second by aerating, and in the third by distilling the bromine into an alkaline solution were then examined for the presence of bromine by our procedure. Negative results in every case established the absence of bromine in the precipitated protein material.

When the fuchsin reagent is treated with iodine, a reddish violet iodo compound soluble in acetone, chloroform, and alcohol is formed. The iodo fuchsin derivative cannot be separated from the bromo compound by our procedure. When less than 20 gammas of iodine are present in 10 c.c. of solution used for the determination, the color of a solution of the iodo derivative in the acetone is the same as that of the bromoderivative. Consequently, if iodine in amounts less than 20 gammas is present in the 10 c.c. of solution being analyzed for bromine, the color obtained by our procedure will be due to iodine as well as bromine, and the results will be too high. When more than 20 gammas of iodine are present, the acetone solution of the iodo fuchsin compound differs in color from an acetone solution of the bromo derivative. Solutions of bromides containing more than 20 gammas of iodine in 10 c.c. being tested cannot be compared with a bromine standard.

VALUES (IN GAMMAS) OBTAINED WHEN 10 C.C. OF SOLUTION ARE ANALYZED FOR BROMINE IN THE PRESENCE OF LESS THAN 20 GAMMAS IODINE

Gammas Bromine in Standard (10 c.c.)	Solutions to Be Tested (10 c.c.) Cone. Bromine in Gammas	Cone. Iodine in Gammas	Apparent Gammas Bromine as De- termined in 10 c.c.
10	5	5	10
10	3	7	10
10	7	3	10

The iodine content of normal blood varies from 4 to 6 gammas per 100 c.c. Since the bromine concentration of blood is at least a hundred times that of iodine, the blood iodine will not materially affect the results for the blood bromine unless the patient is receiving iodine medication.

The fuchsin reagent used in the bromine determination is similar to the well known Schiff's aldehyde reagent, which when treated with aldehydes produces an immediate red color without the addition of persulfuric acid as an oxidizing agent. The absence of such reactions in the case of the protein-free blood filtrate eliminates the possibility of an increase in apparent bromine concentration due to the reaction of aldehydes with fuchsin. The constant values for blood bromine

mides in protein free filtrates kept several hours at room temperature also establish this fact

# BROMINE CONTENT OF PROTEIN FREE FILTRATES KEPT SEVERAL HOURS AT ROOM TEMPERATURE

Fresh filtrate	1 hr	2 hr	4 hr	6 hr	15 hr	24 hr
0.71	-----	---	0.76	--	0.81	----
1.57	1.33	--	-----	1.54	1.42	----
0.92	-----	0.94	0.85	--	0.87	----
0.65	0.70	-----	0.68	---	0.70	0.71

## SUMMARY

1 A colorimetric method for determination of bromine in a protein free filtrate is described

2 The procedure is relatively rapid and the method may be applied generally with the exception of patients receiving iodine medication

3 It has been shown that the blood bromine is entirely in the protein free filtrate and that the protein coagulum contains no bromine

## REFERENCES

- Trances, A G, and Harvey, C O The Micro Determination of Bromine in Blood, *Biochem J* 27 1345, 1933
  - Bier, A, and Roman, W Zur Frage der Bestimmung des "Normalen" Bromspiegels im Blute, *Klin Wchnschr* 12 301, 1933
  - Dixon, T F The Determination of Bromine in Normal Blood, *Biochem J* 28 48, 1934
  - Pincussen, L, and Roman, W Methodische Mitteilungen IX Gemeinsame Bestimmung der Halogene in organischen Substanzen, *Biochem Ztschr* 207 416, 1929
  - Ucko, H The Presence and the Role of Bromine in the Body, *Biochem J* 30 992, 1936
  - Winick, P S, and Smith, A H The Determination of Bromine in Biological Substances, *J Biol Chem* 119 93 1937
  - Damiens, A Sur le dosage de traces de brome dans les matieres organiques, *Compt rend Acad d sc* 171 799, 1920
  - Oppenheimer, E Eine neue Methode zur Bestimmung des Broms in kleinsten Mengen, *Arch f exper Path u Pharmacol* 89 17, 1921
  - Indovina, R Eine neue Methode zur Bestimmung des Broms im Blute, *Biochem Ztschr* 275 286, 1935
  - Yates, E D The Determination of Bromine in Blood, *Biochem J* 27 1763, 1933
  - Conway, L J, and Flood, J C An Absorption Apparatus for the Micro determination of Certain Volatile Substances The Micro determination of Bromide, With Application to Blood and Urine and Observations on the Normal Human Subject, *Biochem J* 30 716, 1936
- Hartner, F Eine einfache Methode zur Bestimmung kleinster Brommengen neben einem grossen Chlorüberschuss, *Ztschr f physiol Chem* 214 179, 1933
  - Möller, K O Zur quantitativen Bestimmung kleiner Bromidmengen in Blut und Harn, *Biochem Ztschr* 245 282, 1932
  - Smith, F F, Duly, M E, and Sloan, D H Distribution of Bromide in Blood Serum and Cerebrospinal Fluid, *Arch Neurol & Psychiat* 33 764, 1935
  - Leipert, T, and Watzlawek, O Die Bestimmung kleinster Brommengen in biologischen Flüssigkeiten, *Ztschr f physiol Chem* 226 108, 1934
  - Guillaumin, C O, and Merejowsky, B Flux, repartition et état physico chimique du brome sanguin chez les sujets normaux et hors des affections mentales, *Compt rend Soc de biol* 113 1428, 1933
  - Doering, H Die Bromwerte des Blutes *Biochem Ztschr* 291 81, 1937
- Denigès, G, and Chelle, L Modification à la technique de la recherche et du dosage du brome ionisé par le reactif fuchsine sulfurique, *Ann chim anal* 23 81, 1918
- Mahmud, W, Fuchs, D M, and Mahmud, N Barrier Between the Blood and the Cerebrospinal Fluid, *Arch Neurol & Psychiat* 20 780, 1928
- Hastings, A B, and van Dyke, H B Studies of Bromide Distribution in the Blood I In Vitro Experiments of Bromide and Chloride Distribution, *J Biol Chem* 92 13, 1931

# PHOTOELECTRIC COLORIMETRY

## II. QUANTITATIVE DETERMINATION OF BLOOD NONPROTEIN NITROGEN, UREA, CREATININE, CREATINE, GUANIDINE, AND AMINO ACIDS\*

DAVID NORTHUP, PH.D., FABRY L. HAWK, M.T., AND  
JEROME E. ANDES, PH.D., M.D., MORGANTOWN, W. VA.

### INTRODUCTION

IN THE first paper of this series<sup>1</sup> is given a brief discussion of photoelectric colorimeters, and it is needless to repeat the same here. It is only necessary to add that we feel that the null-point, two-cell instruments are quite superior to the one-cell, direct-reading types, and, therefore, we have used this type of apparatus. A fairly detailed discussion of the instrument, together with a cent of the same, is given in Fig. 1 of paper I.† In the work outlined below we have not tried to introduce any actually new methods, but have only modified one of the more standard procedures for use with the electrical instrument. We have also given curves (for use with the instrument illustrated), and have pointed out the most satisfactory type of filter for each determination. Wratten (gelatin) light filters, mounted in glass, were used exclusively.

### EXPERIMENTAL

*Use of the Instrument.*—The photoelectric colorimeter (Fig. 1, paper I) is turned on fifteen to twenty minutes before using to allow it to "heat-up," and thereby reach a constant output. The glass cell or cup‡ is then filled with water (or blank of the reagents) placed in the instrument, the milliamperage output of the photoelectric cell set at some definite value (0.40 Ma.), and the galvanometer adjusted to the neutral position with the conduction set at 100. Then the cup is filled with the solution to be tested and the per cent light transmission found, the output of the photoelectric cell being held at 0.40 Ma. all the while. From the graph for this determination, the concentration of the unknown is read off directly.

If filters are used, the current output of the photoelectric cell is too low to be read accurately; consequently the output is adjusted (at 0.40 Ma.) without the filter in place, and the incoming voltage noted. Then by means of a voltage regulator the incoming voltage is held at this value, while the filter is put in place, the instrument adjusted, and the unknown reading taken. The recommended procedures are given in more detail below.

\*From the Departments of Pathology and Physiology, West Virginia University Medical School, Morgantown.

Received for publication, May 19, 1938.

†Manufactured by Eimer and Amend, New York City.

‡These cells are standard glass absorption cells, 1 cm. thick (inside measurement), and hold approximately 18 c.c. of fluid. It is convenient to have at least two such cups, and to use one for the blank (or water) and the other for the unknown solution. The cups must be very clean by washing with soap and water after each determination.

## NONPROTEIN NITROGEN

**Principle**—This is essentially the method of Folm and Wu,<sup>2</sup> using a selenium digestion mixture.<sup>3, 4</sup> The tungstic acid blood filtrate is digested with acid and then nesslerized. From the value for the light transmission of the colored solution, the concentration of nonprotein nitrogen is obtained directly from a graph.

**Procedure**—Prepare a protein free blood filtrate by adding one volume of blood to seven volumes of water, shaking and waiting three to four minutes to allow the corpuscles to lake, and adding one volume of  $\frac{2}{3}$  normal sulfuric acid\* and one volume of 10 per cent sodium tungstate. Shake and filter or centrifuge at once. The filtrate should be crystal clear.

Measure 5 cc of this filtrate into a nonprotein nitrogen tube (pyrex test tube graduated at 35 and 50 cc), and add 1 cc of selenium digestion mixture. By means of a microburner, heat the solution under a hood until white sulfur trioxide fumes begin to fill the tube. Then reduce the size of the flame greatly, and continue heating (never at any time allowing the flame to touch the tube above the level of the liquid) until the solution is clear. A slight amount of brown flakes in the top of the tube is of no harm, as this material floats on the surface of the liquid through the rest of the procedure. After cooling for a few minutes, dilute with water nearly to the 35 cc mark, and add 15 cc of diluted Nessler's solution. Dilute to 50 cc with water, mix, and read at once in the photoelectric colorimeter, after first setting the instrument with the cup filled with a blank of the reagents (35 cc of water + 15 cc of Nessler's) and using a No. 47 Wratten filter. A current of 0.40 Ma is used (without the filter in place). A voltage regulator must be employed if the voltage is not constant. The nonprotein values are read directly from the graph in Fig. 1. If the value is above 60 to 80 mg, the analysis had best be repeated, using a smaller amount of the filtrate. If speed is necessary, such a sample may be saved by adding to the nesslerized unknown one or more equal parts of water (containing 15 cc of Nessler's + 1 cc of digestion mixture per 50 cc of volume), and redetermining the light transmission.

\*Prepared by adding 186 cc of concentrated sulfuric acid (94 per cent sp gr 1.84) to water and making the volume up to 1000 cc.

†Note that the reagents are added in the reverse order. This gives a more complete precipitation of the proteins and the material can be filtered at once. This method of precipitation seems to be necessary in the determination of blood creatine.<sup>5</sup>

‡This is slightly different than either of the mixtures of Campbell and Hanna<sup>3</sup> or Rels and Powers.<sup>4</sup> It consists of 100 mg of selenious acid (selenium dioxide) added to 200 cc of a 3:1 mixture of concentrated sulfuric and phosphoric acids. It is colorless and quite suitable for photoelectric work.

§The Nessler's solution is prepared from a mixture of the double iodide and water the dilutions being different for urea and nonprotein nitrogen determinations.

**Double Iodide**—To 75 gm of potassium iodide and 110 gm of iodine in a 250 cc flask add 50 cc of water and 70 to 75 gm of metallic mercury. Shake the flask continuously and vigorously until the dissolved iodine has nearly all disappeared. The solution becomes quite hot. When the iodine color is visibly pale cool in running water. Continue to shake until the red color of the iodine has been replaced by the green color of the double iodide. Do not cool too soon! Separate the solution from the excess mercury by decantation and wash with liberal amounts of distilled water. Dilute the washings and solution to a volume of 2000 cc with water.

**Nessler's Solution for Nonprotein Nitrogen**—Dilute one part of the double iodide with one part of distilled water and add four parts of 10 per cent sodium hydroxide (freshly prepared from C. P. chemicals). This solution keeps several months if stored in a brown glass stoppered bottle.

## UREA NITROGEN

**Principle.**—The blood filtrate is treated with a solution of urease and the color developed by direct nesslerization. This method is a modification of the method of Karr<sup>6</sup> similar to that outlined by the authors.<sup>7</sup>

**Procedure.**—Transfer 5 c.c. of tungstic acid blood filtrate into a test tube graduated at 25 c.c., and add 2 drops of glycerin urease solution\* and 4 drops of phosphate buffer solution.† Incubate in a water bath at 45° to 50° C. for fifteen minutes. Cool, dilute up to about 20 c.c. with water, add 3 c.c. of urea Nessler's solution,‡ and dilute to 25 c.c. Mix and find the light transmission.

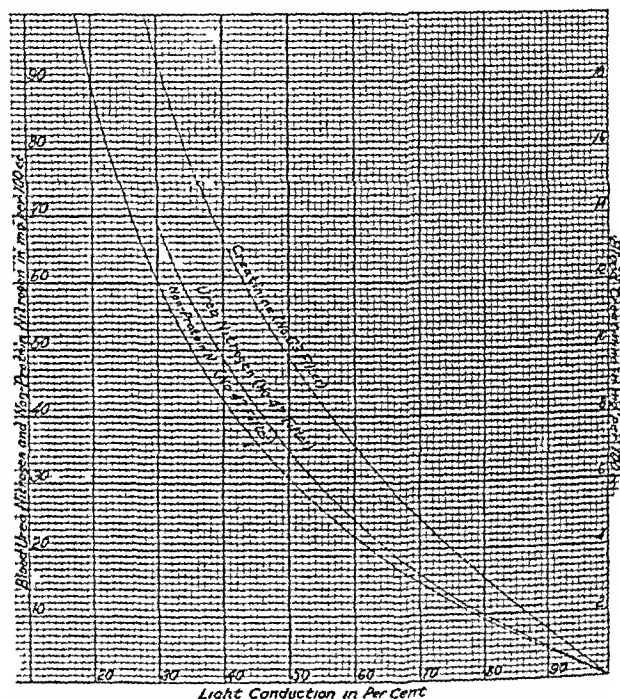


Fig. 1.—Photoelectric light transmission curves for nonprotein nitrogen, urea nitrogen, and creatinine. The type of filter is specified on the curve.

in the photoelectric colorimeter as with the nonprotein nitrogen, after first setting the instrument at 100 with the cup filled with water, Nessler's solution, buffer, and urease (made up to 25 c.c. in volume). A No. 47 Wratten

\***Glycerol Urease Solution.** Shake 15 gm. of permittit with 200 c.c. of 2 per cent acetic acid, decant the aqueous phase, and wash two or three times with distilled water. To the residue, add 50 c.c. of 0.001 normal sulfuric acid and 30 gm. of jack bean meal (Arden Chemical Co.). Shake gently for thirty minutes, add 150 c.c. of glycerin, and mix well. Stand overnight and filter through several thicknesses of gauze. Centrifuge the filtrate. The filtrate is, however, very active and stable. The equivalent of 0.03 to 0.05 c.c. will extract 2 mg. of urea nitrogen completely into ammonia (in a 5 c.c. volume) at 45° to 50° C. in 15 minutes. Two drops are sufficient for most urea determinations. Such an extract will retain its full activity for at least a year at room temperature and exposed to the ordinary room illumination; however, keeping the extract in the refrigerator is to be preferred if this is convenient. See Koch.<sup>8</sup>

†**Phosphate Buffer.** Dissolve 11 gm. of sodium pyrophosphate (15 S.P.) in water, add 10 c.c. of glacial phosphoric acid (85 per cent), and make the volume up to 250 c.c. with water.

‡**Nessler's Solution for Urea.** Mix three parts of the double iodide (see footnote †) with three parts of distilled water. To this mixture add 14 parts of 5 per cent sodium hydroxide (freshly prepared). The solution is best prepared fresh from the double iodide at least every three months.

filter is used, and a photoelectric current of 0.40 Ma (with the filter removed). The urea nitrogen value is obtained directly from the curve in Fig. 1.

For values above 60 mg per 100 cc the determination is best repeated using smaller amounts of filtrate, if necessary the colored solution may be diluted with one or more volumes of water (containing Nessler's solution, urase, and buffer), and the color transmission redetermined.

#### PREFORMED CREATININE

*Principle*—This is essentially the method of Folin and Wu<sup>2</sup> which is very widely used. The red color is developed by treating the blood filtrate with alkaline picrate, and the amount of color determined by measuring the light conduction.

*Procedure*—To one volume (20 cc) of tungstic acid blood filtrate in a 50 cc flask, add one half this amount (10 cc) of alkaline picrate solution,\* mix, allow to stand eight to ten minutes and read the light transmission in a photoelectric colorimeter (using a No. 63 Wratten filter and a current of 0.40 Ma, without the filter). The instrument is previously balanced at 100 with the cup filled with water and the reagent (in the ratio of 2:1). The creatinine is obtained directly from the graph in Fig. 1. For values above 20 mg, the analysis should be repeated with one half the amount of filtrate (diluted with water), in case of a necessity the colored solution may be diluted with an equal amount of water and picrate, and the light transmission redetermined.

#### TOTAL CREATININE AND CREATININ†

*Principle*—Creatinine is determined before and after autoclaving with hydrochloric acid (to convert the creatine into creatinine), and the creatine is calculated from the difference.

*Procedure for Total Creatinine*—Precipitate the blood proteins in the manner given for nonprotein nitrogen‡. To 15 cc of the filtrate in a 25 cc volumetric flask, add 0.3 cc of concentrated hydrochloric acid and autoclave the flask for thirty minutes at 120° C (20 pounds pressure). After cooling, neutralize with 10 per cent sodium hydroxide (using litmus paper as an indicator), and make the volume up to 25 cc. After mixing, determine the creatinine on 20 cc of this solution using the method for preformed creatinine. The value obtained from the graph (Fig. 1) is multiplied by 1.67 to give the total creatinine per 100 cc of blood.

*Calculation of Creatine*—Subtract the preformed from the total creatinine, and multiply this figure by 1.16.

#### BLOOD GUANIDINES§

*Principle*—The guanidines are extracted from blood by blood charcoal, autoclaved to convert creatine into creatinine, and a reddish color developed with a modified Tiegs' reagent.

\* *Alkaline Picrate Solution*. Prepare by adding 5 cc of 10 per cent sodium hydroxide to 25 cc of 1.2 per cent (saturated) picric acid solution. This solution must be prepared fresh for each determination.

† Essentially the method of Folin and Wu as modified by Andes.<sup>3</sup>

‡ It is desirable to add the acid and tungstate in the reverse order to that advised by Folin and Wu as the resultant filtrate usually remains entirely clear when autoclaved.

§ Method of Andes and Myers.<sup>4</sup>

*Procedure.*—The method for preparing the extract given in the original paper was used unchanged. Since the procedure is fairly long, it is not repeated here. To the final extract, add 15 c.c. of water (instead of the 2 c.c. in the original paper) and 5 c.c. of the color reagent,\* and centrifuge three to four minutes to remove the precipitate that always forms at this juncture. Adjust the colorimeter with a blank of water and reagent in the cup, using No. 63 filter and 0.40 Ma. of current from the photoelectric cell (without the filter in place). Now fill the cup with the unknown solution, and read the light transmission within nine minutes from the time the color reagent was added. The guanidine is read directly from the graph in Fig. 2.

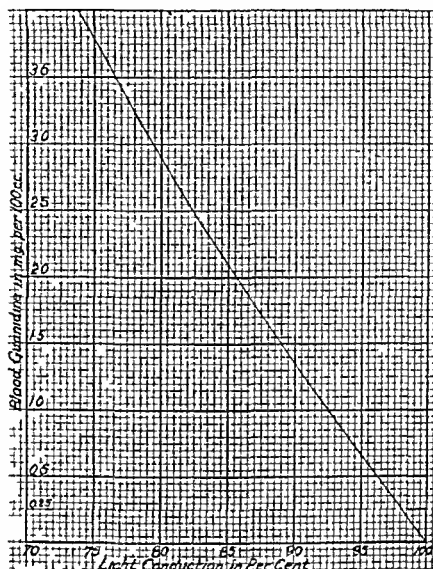


Fig. 2.—Photoelectric light transmission curve for guanidine (No. 63 Wratten filter)

#### BLOOD AMINO ACID NITROGEN†

*Principle.*—Blood of plasma amino acid nitrogen is determined directly on the protein-free filtrate, using a suitable color reagent.

*Procedure.*—To exactly 10 c.c. of tungstic acid blood (or plasma) filtrate in a 25 c.c. volumetric flask, add 2 c.c. of 1.5 per cent borax solution and 2 c.c. of a freshly prepared 0.5 per cent solution of sodium beta-naphthaquinone sulfonate (Eastman Kodak Co.). At the same time prepare a blank of the reagents, using 10 c.c. of distilled water instead of the filtrate. Let both flasks stand in the dark for eighteen to twenty-four hours. Now add to both flasks 2 c.c. of acid formaldehyde reagent,‡ and 2 c.c. of 0.1 molar sodium thio-sulfate solution (not standardized).§ Dilute to 25 c.c. and let stand four to five minutes to allow bleaching of the excess reagent.

\*Details for preparing the reagents are given in the original paper. The color reagent should be made up fresh every day for most accurate work.

†Method of Danielson<sup>10</sup> slightly modified.

‡Acid Formaldehyde Reagent. Mix three volumes of 1.5 normal hydrochloric acid with one volume of glacial acetic acid, and four volumes of 0.15 molar formaldehyde (11.3 c.c. of 10 per cent formaldehyde diluted to 1000 c.c. with water gives a 0.15 molar solution of formaldehyde).

§0.1 Molar Sodium Thio-sulfate. Dissolve approximately 25 gm. of pure crystalline sodium thio-sulfate in water, and make the volume up to 1000 c.c.

Adjust the colorimeter (no filter) at 100 with the blank solution in the cup, with a photoelectric current of 0.40 Ma. Then fill the cup with the unknown solution, and find the light transmission. From the curve in Fig 3 the concentration of amino acid nitrogen is found directly. For values above 20 mg the determination is repeated with a smaller amount of the filtrate. Such high values are only rarely observed and then only in cases of very severe liver injury.

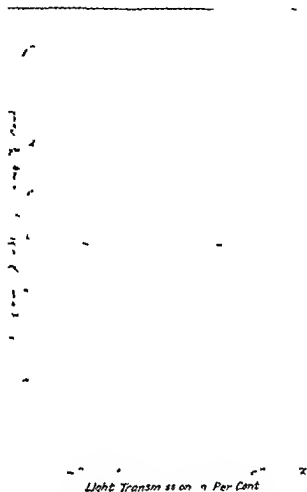


Fig 3—Photoelectric light transmission curve for amino acids (no filter)

#### DISCUSSION

In general, little need be said except to extol the additional accuracy and rapidity with which the photoelectric determinations can be carried out. After one determination alone, one is impressed with both of these points. The lack of necessity for preparing a standard solution is a marked advantage, especially with abnormal blood samples and also with standards that keep poorly. The freedom from eyestrain is a very welcome entity. The main objection is the first cost of the instrument.

The curves for urea, nonprotein nitrogen and creatinine are given only for the lower part of the pathologic range. With the first two substances it is obvious from the graphs (Fig 1) that values above 60 mg are out of the more accurate part of the curve, hence we have not included them in preparing the graphs. The value so rarely exceeds 20 mg for creatinine that it seemed advisable to plot the lower values on a larger scale than to include the entire range. The amino acid curve includes all values except the extremely high ones observed in severe hepatic destruction. In all the determinations the agreement between duplicate determinations is usually quite close.



The most important point (as was also discussed in the first paper of this series) is whether or not these curves can be used by another operator with another (similar) instrument, using other filters. While the best plan is for each worker to construct his own curve, this may not be necessary in routine work. The filters (especially if kept in the dark or in their cases) do not appreciably fade in one year's time. The cups are standard 1 cm. spectroscopic cups, and are constructed very accurately. Since the photoelectric determination is made by the null-point method with a constant current coming from the first cell, we doubt if a different colorimeter will introduce any appreciable error. In fact, we believe that if the values do not need to be more accurate than 5 per cent, the curves listed may be safely used per se.

#### SUMMARY

1. Procedures for determining nonprotein nitrogen, urea, creatinine, creatine, guanidine, and amino acid nitrogen in the blood have been adapted for use in the photoelectric colorimeter.

2. Curves are given to facilitate the use of these methods, and it is believed that the curves may be used by other workers using similar instruments for regular clinical work.

#### REFERENCES

1. Andes, J. E., and Northrup, D. W.: Photoelectric Colorimetry. I. Determination of Blood Iron and Hemoglobin, *J. LAB. & CLIN. MED.* 24: 197, 1938.
2. Folin, O., and Wu, H.: A System of Blood Analysis, *J. Biol. Chem.* 38: 81, 1919.
3. Campbell, W. R., and Hanna, M. L.: The Determination of Nitrogen by Modified Kjeldahl Methods, *J. Biol. Chem.* 119: 15, 1937.
4. Reis, F., and Powers, H. H.: Selenium as a Catalyst in the Determination of Nonprotein Nitrogen of the Blood, *J. LAB. & CLIN. MED.* 20: 1204, 1935.
5. Andes, J. E.: The Determination of Blood Creatine Using 3, 5-Dinitrobenzoic Acid as a Color Reagent, *Am. J. Clin. Path.* 8: 12, 1938.
6. Karr, W. G.: A Method for the Determination of Blood Urea Nitrogen, *J. LAB. & CLIN. MED.* 9: 3, 1924.
- Looney, J. M.: The Determination of Blood Urea Nitrogen by Direct Nesslerization, *J. Biol. Chem.* 88: 189, 1932.
7. Hawk, F. L., and Andes, J. E.: A Simplified Method for the Determination of Blood Urea, *Am. J. Clin. Path.* 8: 153, 1938.
8. Koch, F. C.: A Stable and Convenient Urease Reagent and a Modified Blood Urea Method, *J. LAB. & CLIN. MED.* 11: 776, 1926.
9. Andes, J. E., and Myers, V. C.: Guanidine-like Substances in Blood. I. Colorimetric Estimation and Normal Values, *J. LAB. & CLIN. MED.* 22: 1147, 1937.
10. Danielson, I. S.: Amino Acid Nitrogen in Blood and Its Determination, *J. Biol. Chem.* 101: 505, 1933.

# A SIMPLE RAPID METHOD FOR THE DETERMINATION OF TOTAL PROTEIN AND ALBUMIN CONCENTRATIONS IN BLOOD PLASMA, SERUM, OR OTHER BODY FLUIDS\*

DAVID A. RATIND, M.D., SAN FRANCISCO, CALIF

THE concentrations of plasma total protein and albumin can be determined simply and quickly by centrifugalization after precipitation by a modified Folin Wu technique. The results so obtained are more than adequate for clinical purposes, and can be readily carried out by the doctor himself or his office nurse. These methods were developed in response to the needs for methods simplifying the office management of Bright's disease, but can be applied as well to other body fluids and to plasma or serum in other diseases in which knowledge of protein concentration is necessary.

Of fundamental importance in these methods is the use of a centrifuge which runs at a constant speed (3,500 r.p.m.) for a constant time, regardless of voltage fluctuations, and with minimized temperature rise (due to "wind age"). Such a centrifuge has been constructed and is now on the market. Furthermore, centrifuge tubes molded of transparent unbreakable plastic material are now available, these tubes are not only graduated identically and accurately, but also have flat bottoms so that the radial distance, and therefore the force of centrifugalization is always constant. The methods to be described have all been carried out with the above equipment†

## METHODS

*Principle*—One reads the volume of protein precipitated from 1 cc of plasma (or other body fluid) after the addition of sulfuric acid and sodium tungstate and after centrifugalization. Under certain standard conditions, 30 mg of protein yield 1 cc of packed precipitate, so that the volume of precipitate multiplied by 3 gives the protein concentration directly in per cent.

*Reagents*—1/10 N sulfuric acid, 10 per cent sodium tungstate solution, and saturated ammonium sulfate solution.

## TOTAL PROTEIN

*Procedure*—Place 1 cc of plasma into an accurately graduated 15 cc centrifuge tube. Add 8 cc of 1/10 N sulfuric acid, mix by inversion two or three times. Add 1 cc of 10 per cent sodium tungstate solution, and mix by inversion twice. Centrifuge as soon as possible at 3,500 r.p.m. for exactly five minutes. The flat-topped precipitate packs cleanly, and its volume is easily read.

\*From the Department of Medicine, Stanford University School of Medicine, San Francisco.

This work was aided by a grant from the Rockefeller Foundation.

Received for publication October 15, 1938.

†The centrifuge and tubes are supplied by the Electric Manufacturing Co., 34 Dore St., San Francisco.

*Calculation.*—The volume of packed protein precipitate in cubic centimeters multiplied by 3 gives the total protein concentration directly in per cent.

Rarely the volume of precipitate is 2.5 c.c. or more (7.5 per cent or more); when this occurs, the determination should be repeated, using 0.5 c.c. of plasma.

TABLE I  
PLASMA PROTEIN CONCENTRATIONS IN NORMAL INDIVIDUALS, GRAMS PER 100 C.C.

	TOTAL	ALBUMIN	GLOBULINS
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
	8.1	3.6	4.5
	7.2	4.5	2.7
	7.2	3.9	3.3
	6.9	5.4	1.5
	6.9	4.6	2.3
	6.9	4.6	2.3
	6.9	4.6	2.3
	6.9	4.5	2.4
	6.9	4.2	2.7
	6.9	3.9	3.0
	6.8	4.7	2.1
	6.6	4.1	2.5
	6.5	4.7	1.8
	6.5	4.6	1.9
	6.5	4.2	2.3
	6.3	4.5	1.8
	6.3	4.2	2.1
	6.3	3.9	2.4
	6.0	4.8	1.2
	6.0	4.5	1.5
Means	6.7	4.4	2.3
Means	7.0	4.4	2.6
(Peters and Van Slyke <sup>2</sup> )			

TABLE II  
PLASMA PROTEIN CONCENTRATIONS IN CERTAIN PATIENTS, GRAMS PER 100 C.C.

	TOTAL	ALBUMIN	GLOBULINS	DIAGNOSIS
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	
	6.0	2.6	3.4	Renal edema
	6.0	2.1	3.9	Renal edema
	5.6	2.1	3.5	Renal edema
	5.6	1.8	3.8	Renal edema
	5.3	2.0	3.3	Renal edema
	5.1	2.7	2.4	Renal edema
	4.8	3.1	1.7	Renal edema
	4.7	3.6	1.1	Renal edema (marked lipemia)
Means	5.4	2.5	2.9	Renal edema
	6.9	4.2	2.7	Cardiac edema
	6.9	3.6	3.3	Cardiac edema
	6.2	3.3	2.9	Cardiac edema
	5.9	3.0	2.9	Cardiac edema
	5.7	3.6	2.1	Cardiac edema
Means	6.3	3.5	2.8	Cardiac edema
	9.0	1.8	7.2	Multiple myeloma

8.5 c.c. of 1/10 N sulfuric acid, and 1 c.c. of 10 per cent sodium tungstate. The packed volume of precipitate should then be multiplied by 6.

#### ALBUMIN

*Separation.*—To 2 c.c. of plasma, add 2 c.c. of saturated ammonium sulfate solution, and mix by several inversions. Centrifugalization at 3,500 r.p.m. for fifteen minutes gives a clear or nearly clear supernatant fluid. Complete clear-

ing is more easily obtained by centrifugalization of two tubes, each with 2 c.e. of the plasma-ammonium sulfate mixture, or better yet, of four tubes each with 1 c.e. During this step, it is essential to allow the centrifuge to continue revolving freely after the current shuts off, for even gentle braking may cause turbidity in the albumin fraction. (At all other steps in these methods, the machine may be braked if desired.) The usual minor degrees of cloudiness have no appreciable effect on the determination of albumin, nor does the milky fluid of lipemic plasma.

*Procedure.*—The supernatant fluid is drawn off by means of a capillary pipette. Place 2 c.e. (equivalent to 1 c.e. of the original plasma) in an accurately graduated centrifuge tube, and add 7 c.e. of 1/10 N sulfuric acid; a precipitate forms, but immediately disappears on mixing by inversion two or three times.

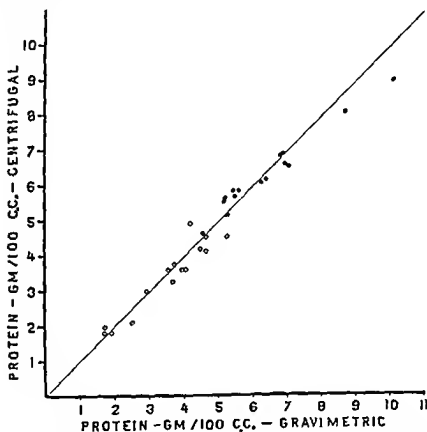


FIG. 1.—Comparison of results by the new methods with determinations by a standard gravimetric method. The line indicates perfect correlation. The dots indicate total protein, the circles albumin concentrations.

Add 1 c.e. of 10 per cent sodium tungstate, and invert twice. Centrifuge as soon as possible for exactly five minutes at 3,500 r.p.m. The precipitate of albumin packs cleanly.

*Calculation.*—The volume of packed precipitate in cubic centimeters multiplied by 3 gives the albumin concentration directly in per cent.

## RESULTS

The concentrations of total protein and albumin by the simple methods given above have been checked against results obtained by a standard gravimetric method<sup>1</sup> on the same samples of plasma (2 mg. of potassium oxalate per c.e. of blood) and albumin solution (fifteen samples each). Fig. 1 gives the comparison of results by the two methods.

The average error was  $\pm 0.3$  gm. per 100 c.e. Thirteen determinations were less than 0.25 gm. in error (of these, 9 erred less than 0.1 gm.), and 13 more were

within 0.5 gm. of the correct concentration. Three other determinations (on normal or high concentrations) deviated by 0.6, 0.6, and 0.7 gm. The thirtieth determination, while 9.0 instead of 10.1 gm./100 e.e., was done only routinely in the course of standardizing these methods, and led to the diagnosis of a previously unsuspected multiple myeloma, later confirmed by x-ray studies made because of the high total protein and low albumin concentration determinations.

In addition to the gravimetric standardization cited above, these methods have been used on 20 determinations of total plasma protein and albumin concentrations in normal individuals; 8 in patients with edema due to chronic glomerular nephritis, and 5 in patients with edema because of congestive heart failure. The results are given in the tables, and are those well known to be typical of these clinical conditions. The normal averages agree very well with those given by Peters and Van Slyke.<sup>2</sup>

#### OTHER BODY FLUIDS

These methods have been applied to an exudate (tuberculous pleural effusion), with results of 5.5 per cent for total protein (5.3 per cent by the gravimetric method) and 3.3 per cent for albumin concentration. Transudates (ascites with portal cirrhosis) have also been satisfactory, giving concentrations of 1.5 per cent, 1.2 per cent, and 0.9 per cent for total protein with 0.6 per cent, 0.45 per cent, and 0.45 per cent for the respective albumin fractions. (The first ascitic fluid was checked against the gravimetric method, which gave concentrations of 1.2 per cent and 0.6 per cent for total protein and albumin.) Preliminary work has shown that urinary and spinal fluid protein concentrations may also be determined by modifications of these methods.

#### DISCUSSION

The chief new features of the above methods are (a) the use of the Folin-Wu reagents, which give a more voluminous and more accurately readable precipitate than do other protein precipitants, and (b) the use of equipment which results in great constancy of centrifugalization. By their combined use, the methods become simple and are quickly carried out, and all but the most simple calculations are eliminated.

All of the above determinations have been made at a room temperature between 20° and 25° C. In this range, temperature does not appreciably alter the volume of packed precipitate from a given amount of protein. With warmer temperatures, the packed precipitate is less, and with colder temperatures greater, than when working at 20° to 25° C. Enough data have been obtained to permit the use of approximate correction factors. At 10° C. the observed protein concentration will be about 115 per cent of the correct concentration; at 15° C., 110 per cent; at 30° C., 90 per cent; at 35° C., 85 per cent. The methods are, of course, more accurate if carried out in rooms at 20° to 25° C. Specimens taken from an icebox should first be permitted to reach room temperature.

Great care should be taken to mix thoroughly the plasma and sulfuric acid before adding the tungstate, or the results may be too low; during the two or three inversions one must allow the contents of the conical tip to drain.

well. The sodium tungstate solution should be reasonably fresh and neither too acid nor too alkaline.<sup>4</sup>

#### SUMMARY

Simple, quick, and clinically adequate methods have been described for the determination of total protein or albumin concentration in plasma, serum, or other body fluids. They may be carried out in the doctor's office by his nurse or by himself.

Thanks are due to Evelyn Barrett BS for the gravimetric determinations of protein and albumin concentrations.

#### REFERENCES

1. Barnett C W, Jones R B and Cohn R B. The Maintenance of a Normal Plasma Protein Concentration in Spite of Repeated Protein Loss by Bleeding. *J Exper Med* 55: 683, 1932.
2. Peters J P and Van Slyke D D. *Quantitative Clinical Chemistry*, Vol I. Interpretations. Baltimore, 1931, Williams & Wilkins Co. pp 653-724.
3. Peters J P, and Van Slyke D D. *Quantitative Clinical Chemistry*, Vol II. Methods. Baltimore, 1932. Williams & Wilkins Co. p 66.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**PNEUMOCOCCI, Microscopic Precipitin Test for Typing, Schaub, I. G., and Reld, R. D.**  
J. A. M. A. 111: 1285, 1938.

*Preparation of the Antigen.*—Cultures of pneumococci from blood, spinal fluid, or other sources taken during life or at autopsy may be grown in meat infusion broth containing a few drops of serum or ascitic fluid, incubated at 37° C. for from six to eighteen hours. The culture is lysed by the addition of two drops of a 10 per cent aqueous solution of sodium desoxycholate (containing 1:50,000 merthiolate to prevent the growth of molds) per cubic centimeter of culture. In from fifteen to thirty minutes the organisms are dissolved, and tests have shown that the lysate is sterile. The antigen is now ready for use. In some instances an excess of sodium desoxycholate in the presence of serum will cause the culture medium to gel. This may be liquefied by warming slightly without decreasing the efficiency of the test.

*Preparation of the Typing Serum.*—For making this test, horse antipneumococcus serum such as is used for macroscopic agglutination tests, diluted 1:10 in physiologic solution of sodium chloride, is used. Such dilutions have been found satisfactory after refrigeration for as long as six weeks.

Rabbit antipneumococcus serum as prepared for the Quellung test, diluted 1:1 in salt solution, has been used, but the reaction takes place more slowly, sometimes requiring from one to two hours for marked precipitation to take place. The reaction with the diluted horse serum takes place rapidly, and readings can be made after from fifteen to thirty minutes. By adding 1 per cent aqueous methylene blue to the horse serum the reading of results is facilitated, although the dye is not essential to the test. Dye is already present in the rabbit Quellung serum.

Attempts were made to place the organisms into groups by means of commercially prepared Quellung grouping sera or by mixing the typing sera prepared from horses in proportions based on the titer of the species antiserum, but results obtained were not sufficiently uniform to be considered satisfactory.

*Technique of the Test.*—The Petri dish-hanging drop technique described by Brown is used for making the test. A disk of moist filter paper is placed in the lid of the dish. The bottom of the dish is ruled on the bottom surface with a wax or diamond pencil, providing sixteen 12 mm. squares. In the performance of the test two Petri dishes are required for each organism to be typed. In one dish the antigen is set up against each of the fifteen types of antiserum (diluted), the sixteenth square being used for an antigen-salt control. In the second dish the remaining type antisera are set up. It has been found most practical to add one platinum loopful of the antigen to each of five squares and mix them with an equal amount of the appropriate typing sera, and then to proceed with the next five squares in the same manner. This prevents drying of the loopful of antigen before it is possible to add the antiserum, as is likely to happen if the antigen is placed in all fifteen squares before the antisera are added. A 2 mm. loop of platinum rather than one of a platinum substitute is recommended. The loop should be thoroughly flamed and cooled each time before being introduced into the serum.

*Reading of Results.*—The mixtures of serum and antigen are held from fifteen to thirty minutes, and readings are made with the 16 mm. objective of the microscope, the light from the condenser being allowed to pass through the moist filter paper. Gentle rotation of the plates hastens the formation of large clumps of precipitate. The appearance of large clumps of precipitate indicates a positive reaction. Negative results are indicated by the absence of such clumping. Doubtful or questionable results are seldom encountered but can be eliminated by comparison with the control and a known positive. In using this technique

for the first few times it is suggested that known positives and negatives be set up as controls to familiarize the worker with typical reactions.

Infrequently, though occasionally, cross reactions occur. These may be distinguished from typical positive reactions with a little experience. Such a doubtful cross reaction is shown in B.

There appears to be no advantage in incubation or refrigeration of the mixtures.

#### **SEDIMENTATION RATE, Normal, in Open Pulmonary Tuberculosis, Banyai, A. L., and Caldwell, E. Am Rev Tuberc 38 491, 1938**

A study of the relationship between positive sputum and the sedimentation test in 5,274 cases of pulmonary tuberculosis is reported. It was found that in 21 per cent of these patients normal sedimentation rate and positive sputum were present at the same time. The symptomatology and simultaneous roentgenologic findings corroborated the presence of an active parenchymal pulmonary tuberculosis in the great majority of instances.

The analysis of the clinical material revealed not only the possible coexistence of an active pulmonary tuberculosis and normal sedimentation velocity, but also that single or multiple cavities may coexist with a normal sedimentation rate.

The most plausible explanation of the varying rates of the sedimentation of the red blood cells is a disturbance of the colloidal balance of the blood. As a rule, there is a parallelism between the activity of the tuberculous process and the colloidal balance of the blood. Consequently, tissue destruction or tuberculous toxemia that upsets the equilibrium of the serum proteins is usually recognizable by an increased sedimentation speed.

The exceptions to this rule, as found by the authors and by others, can be explained by the hypothesis that there are certain cases of pulmonary tuberculosis in which the colloidal balance may remain normal during the active stage of the disease or it may return to normal prior to cessation of activity of the tuberculous process.

The authors are of the opinion that, although these exceptions do not invalidate the value of the sedimentation test, its limitations should be recognized and, therefore, a normal sedimentation rate should not be considered an infallible evidence of the absence of an active pulmonary tuberculosis.

#### **SYPHILIS, Frequency With Which Syphilitic Lesions Are Encountered in Postmortem Examinations, Bell, E. T. Arch Path 26 882, 1938**

In a survey of 27,872 post mortem examinations it was found that definite syphilitic lesions were noted in 2.77 per cent, and in approximately 2.5 per cent syphilis was shown to be the major cause of death. About 3.38 per cent of stillborn infants were syphilitic, and congenital syphilis caused about 2.63 per cent of the deaths in infants under 1 year of age. Congenital syphilis was a rare cause of death in infants more than 1 year of age.

Syphilis was an infrequent cause of death in persons dying in the second and third decades of life. The maximal mortality from this cause occurred in persons in the fourth, fifth, and sixth decades of life.

Acquired syphilis, in its lethal forms at least, was about twice as frequent in males as in females.

The major forms of lethal syphilis were syphilitic aortitis and neurosyphilis. The age distribution of these two forms was about the same.

#### **TUBERCLE BACILLI, A Medium for the Culture, Isolation and Dissociation of, Steenken W., Jr., and Smith, M. M. Am Rev Tuberc 38 514, 1939**

A modification of Hohn's medium has been recommended. It differs from Hohn's in that it does not contain alumina; it contains only half the amount of asparagin, licmoid is substituted for malachite green to impart a dark blue background instead of the light green produced by malachite green; it has an adjusted final pH of 6.2 as contrasted to Hohn's, which is unadjusted but usually has a final pH between 7.1 and 7.3.



It has also been shown that the modified medium is preferable to Corper's medium for colony study, since it eliminates marked atypical structures.

The use of the Fisher culture tube No. 14-927 is recommended for colony study.

The formula of the medium follows:

Distilled water	500 c.c.
Sodium phosphate	1.5 gm.
Potassium dihydrogen phosphate	2.0 gm.
Magnesium sulfate	0.3 gm.
Magnesium citrate	1.25 gm.
Asparagin	1.50 gm.
Glycerin	60 c.c.

*Preparation of the Medium.*—The salts are dissolved in 500 c.c. of distilled water at a temperature of about 80° C. The glycerin is added last and the mixture is sterilized in the autoclave at 10 pounds pressure for twenty minutes. This is the stock synthetic solution. To every 50 c.c. of synthetic stock used, 4.4 c.c. of a 1.0 per cent laemoid (this is made up in a 50 per cent alcoholic solution) solution is then added; 163 c.c. of whole egg mass is added, making a proportion of synthetic dye egg mixture of 1:3 and the final concentration of laemoid 1:5,000. This mixture is filtered through sterile gauze and the filtrate adjusted with N/1 hydrochloric acid, preferably with the aid of a potentiometer, to pH 6.0. The filtrate must be shaken thoroughly while adding the acid. It is now placed in tubes and inspissated for one and a half hours at 85° C. on two successive days. (The pH of the medium after inspissation becomes 6.2.) The medium may turn yellow on inspissation, especially in the deeper portions. This change in color is temporary, for on cooling, the medium assumes its original blue color.

It has been the desire of most originators of media for the cultivation and isolation of tubercle bacilli to add a dye bacteriostatic for all microorganisms other than the tubercle bacillus. It has been the authors' experience that practically all dyes incorporated in media to prevent the growth of secondary microorganisms have some inhibitory effect upon the growth of the tubercle bacillus. This inhibition is most pronounced when there are but few tubercle bacilli present in the material to be cultured.

It has also been observed that the digestion of the specimen is the most important factor in obtaining uncontaminated cultures. Careful digestion means vigorous shaking of the specimen with the proper concentration of sodium hydroxide or sulfuric acid until it is homogeneous and quite fluid, then incubation for one hour at 37.5° C.

It may be necessary to shake some specimens occasionally during this period of incubation to insure breaking up of clumps and allowing the digestion fluid to penetrate all of the material. Whatever time is spent in the preparation of the specimen for culture is well compensated for in the number of uncontaminated cultures obtained, regardless of the dye incorporated in the medium.

**STREPTOCOCCI, Hemolytic, Precipitin Test Applied to Melbourne, Butler, H. M. M.**  
J. Australia 11: 501, 1938.

The results are reported of an examination of 113 strains of hemolytic streptococci isolated in Melbourne from human sources. Both group precipitin and biochemical reactions were studied.

With Lancefield's technique for the group precipitin test 25 strains could not be allotted with certainty to any one group, since 7 failed to react with any of the group sera, and 18 gave pronounced cross reactions. When formamide extracts were prepared from these strains according to the method of Fuller, all but one could be allotted to one or other serological group. It had been noted, however, when the Lancefield tests were being carried out, that most of these strains of hydrochloric acid extracts which reacted with group A anti-serum and also with one or more of the other sera differed from the strains which had reacted with the group A serum alone, in that they grew on 40 per cent ox bile blood agar plates and that in the primary cultures on blood agar plates the colony form was different. Further

these atypical strains were not associated with signs of serious infection. In this paper those strains which belonged to group A, according to the formaldehyde method but which gave cross reactions with Lincefield's method, and which in addition were atypical either as regards tolerance for bile or colony form, have been referred to as atypical members of group A.

Of the 113 strains, 70 were typical members of group A, 12 were classed as atypical group A strains, 10 belonged to group B, 9 to group C, and 8 to group G. One failed to react with any of the four groups of antisera used.

Although the majority of strains associated with severe infections were typical members of group A, a group B and a group C strain respectively were the only organisms cultivated from the blood of two patients who were gravely ill. Of 46 strains associated with severe infections, 42 were typical members of group A, of 37 strains from patients with moderately severe infections, 24 were typical members of group A while 30 mild cases yielded only four such strains.

The clinical condition of the patients with puerperal fever cultures from whose vaginal swabbings yielded atypical group A streptococci differed from that of the patients infected post partum with typical group A strains.

#### SILICOSIS, Study of, Matz, P. B. *Am J M Sc* 196 543, 1938

Silicosis is the chief occupational disease in this country. The seriousness of silicosis is due to the fact that it is conducive to the inception of pulmonary tuberculosis. Tuberculous silicotics are a source of danger to their fellow workers as well as to members of their families, because of the ease with which tuberculous infection is transmitted. The disease results in a great economic loss to society.

A study of the classification of siliceous disease in a group of 167 veterans showed that 30 per cent were classified as first, second, or third degree silicosis, 11 per cent were cases of silicosis with nontuberculous infection, 42 per cent were cases of silicosis with tuberculosis, 16 per cent were classified as silico tuberculosis, and 1 case was diagnosed as asbestosis.

The various siliceous occupations act differently in causing silicosis. The differences are due to a number of factors, such as the silica content of the dust, the size of the silica particles, the variation in the time of exposure, the presence of organic or nonsiliceous dusts mixed with silica, and the probability of the existence of an individual susceptibility.

In the group of cases studied, it was found that the most frequent complications and coexisting diseases were pulmonary tuberculosis, cardiovascular disease, emphysema, pulmonary infections, bronchiectasis, chronic pleurisy, and dental diseases.

In a study of the classification of the 97 cases of silicosis complicated by tuberculosis, it was found that in 11 per cent the tuberculous disease was classified as incipient, in 32 per cent it was moderately advanced, and in 57 per cent far advanced.

The cardiovascular system may show evidence of discrete silicosis. The abnormal cardiac changes are characterized by a progressive dilatation and hypertrophy due to obstruction of the pulmonary circulation, the result of extensive fibrosis of the lungs. Of 120 living silicotics, 50 (41.7 per cent) gave evidence of disease of the heart or blood vessels, of this number 28 showed the presence of cardiac hypertrophy, dilatation, or a combination of the two cardiac conditions, 16 showed the presence of fibrosis of the myocardium, and 21 gave evidence of arteriosclerosis. Of 24 deceased silicotics on whom no post mortem examination was done, 13 showed the presence of cardiovascular disease, of which number 6 were cases of cardiac dilatation, or a combination of hypertrophy and cardiac dilatation. In a series of 23 autopsies on silicotic subjects, reported elsewhere by the writer, hypertrophy or dilatation of the heart or a combination of the two conditions was found in 18 instances. These cardiac conditions were found in the early as well as in the advanced stages of silicosis, also in silico tuberculosis.

While tuberculosis is the principal complicating infection in silicosis, other infections such as pneumonia, lung abscess, and bronchiectasis, are frequently encountered. Upper and lower pulmonary tract infections were found in 56 (34 per cent) of the group of 167 silicotics,

there were 39 cases of chronic bronchitis, 11 cases of bronchiectasis, and 6 cases of chronic pharyngitis. Chronic pleurisy was present in 66 (40 per cent) of the number; this condition is ordinarily not found in uncomplicated silicosis, but in silicosis with a complicating infection.

**LEUKOCYTES, The Phagocytic Activity of Human, With Special Reference to Their Type and Maturity, Hertzog, A. J. Am. J. Path. 14: 595, 1938.**

The phagocytic activity of the leucocytes from 17 cases of leucemia, 3 cases of infectious mononucleosis, 4 cases of lymphocytosis, and 2 cases of eosinophilia were investigated. Staphylococci and streptococci were used as test particles.

The mature polymorphonuclear neutrophiles showed the greatest amount of phagocytosis both as to the number of bacteria ingested per cell and as to the percentage of cells engulfing bacteria. The monocytes, eosinophiles, and metamyelocytes were also actively phagocytic.

The phagocytic activity of the myelocytes, promyelocytes, leucoblasts, and myeloblasts appears to be directly proportional to the maturity of the cell as there was a marked decrease in phagocytosis in the more immature forms. An exception was found in the histoid stem cell and histoid monoblast of leucemic reticulo-endotheliosis, as these immature cells showed an unusual degree of phagocytosis.

Phagocytosis was observed in a small per cent of mature lymphocytes. The degree of phagocytosis increased as the cell grew larger, with a corresponding increase in cytoplasm. The leucocytoid lymphocyte of infectious mononucleosis showed the great phagocytic activity. Hence, lymphocytes in their prephagocytic stage may occasionally show phagocytosis under experimental conditions.

**PNEUMONIA, Recent Studies on Experimental Lobar, Robertson, O. H. J. A. M. A. 111: 1432, 1938.**

By implanting pneumococci suspended in a starch-broth paste into the terminal air sacs, it has been possible to produce in the dog a lobar pneumonia which closely resembles that which occurs in the human being.

The pneumococci are dispersed from the locus of implantation principally by the edema fluid of the early lesion, which spreads peripherally through the contiguous air passages and the pores of Cohn.

The evolution of the inflammatory lesion of the canine disease is essentially the same as that observed in human lobar pneumonia.

At the time of recovery a striking histologic change occurs in the pulmonary lesions of both man and the dog. This consists in the transformation of certain of the fixed tissue cells into free macrophages which engulf and destroy the pneumococci much more effectively than do the polymorphonuclear leucocytes. The macrophages are dependent on opsonins for their antipneumococcal activity.

The mechanism of recovery appears to be of dual nature, consisting of a generalized process, which acts to localize the infection and prevent or control bacteremia, and a local process, the macrophage reaction, whereby the lung is enabled to rid itself of the invading microorganisms. If both processes are effective, recovery results. If either one fails, death ensues.

One attack of experimental pneumonia confers an increased resistance to subsequent infection which lasts for many months. The basis of such immunity seems to reside in the greatly accelerated macrophage reaction characterizing the recurrent lesions.

After recovery a high degree of local immunity can be demonstrated in the involved lobes; it persists only as long as the macrophages are present in the alveoli. However, the duration of the local immunity is greatly prolonged by repeated infection in the same locus. Under such circumstances a residual mobilization of macrophages is observed in the tissues of the lung.

# *The Journal of Laboratory and Clinical Medicine*

VOL 24

FEBRUARY 1939

No 5

## *CLINICAL AND EXPERIMENTAL*

### PERFORATED PEPTIC ULCER\*

#### ITS DIFFERENTIATION FROM ACUTE PANCREATITIS BY BLOOD DIASTASE DETERMINATION

J G PROBSTEN, M D, P A WHEELER M D AND S H GRAY, M D  
ST LOUIS MO

THE differential diagnosis of acute disorders of the upper abdomen frequently presents clinical problems of the utmost perplexity. The similarity in the clinical details which require evaluation renders accurate and sometimes even presumptively accurate diagnosis difficult. Any laboratory aid which might help in these cases should be a welcome supplement to the clinical judgment of the surgeon.

The use of blood and urinary diastase determinations in the differential diagnosis of acute upper abdominal disorders has lacked the widespread utilization which it deserves. The neglect of this test, which we consider to be of great worth, can probably be attributed to inaccuracies and laboriousness of the analytical methods, making the clinical application of the test at times impractical. The presence of a starch splitting enzyme in the blood and urine has been known for many years. All of the analytical methods were able to show the relatively enormous increases in the blood and urinary diastases always associated with acute pancreatic disorders. It is the small increases in diastase values and the subnormal values which have been difficult to detect by the usual methods.

Most investigators have adopted the starch sugar reduction method of quantitative diastase estimation. Certain discrepancies in the method have been detected by Somogyi,<sup>1</sup> and he has devised a method of determination,

\*From the Laboratory of the Jewish Hospital and the Snodgrass Laboratory, City Hospital, St. Louis.

This work aided by the Florence C. and David May Fund.

Received for publication June 2, 1938.

substituting starch paste for soluble starch, which is remarkable for its simplicity and rapidity of performance, and which, after checking and rechecking thousands of blood and urinary determinations, has been found to give accurate and reproducible results. Normal blood diastase values obtained by this method range between 80 and 150, meaning that 1,000 c.c. of blood plasma incubated with starch paste for thirty minutes under standard conditions produce reducing substances that reduce as much copper as 80 to 150 mg. of glucose.

Using this method, careful statistics are being compiled in conjunction with several thousand cases of all types at the Jewish and City Hospitals in an endeavor to apply the results clinically. It can be stated that we have found the diastase test of great value in the differential diagnosis of upper abdominal affections.

In a recently published report,<sup>2</sup> we confirm the importance of the elevation of diastase values obtained in cases of acute upper abdominal pain presumptively of pancreatic origin. Twenty-one cases of so-called "transient acute pancreatitis" are described in which the blood diastase values at times reached 2,500. The report concludes that cases of acute pancreatitis are more numerous than is commonly supposed, that the majority are of a transient nature and recover within a few days, and that the routine use of blood diastase estimations as an emergency diagnostic procedure will uncover cases of transient pancreatitis that are frequently thought to be biliary colic, perforating ulcer, acute appendicitis, coronary disease, etc.

The preliminary results of a statistical analysis now being prepared reveal that the circulating diastase usually drops to a subnormal level in cases of impaired liver function, including cases of biliary tract disease, cirrhosis, primary and secondary carcinoma, malignant disease, chronic passive congestion, abscess, etc.

TABLE I  
BLOOD DIASTASE IN PERFORATED PEPTIC ULCER

CASE	PREOPERATIVE	POSTOPERATIVE	INVOLVEMENT OF PANCREAS
1	214	130	Yes
2	200	150	Yes
3	600-402-150-90	No operation	Yes
4	198		Yes
5	84		No
6	107	73	No
7	67	40-40-76	No
8	31		No
9	59		No
10	90	Not determined	No
11	72	Not determined	No
12	30	Not determined	No
13	60	Not determined	No
14	78	Not determined	No
15	36	Not determined	No
16	84	Not determined	No
17	67	46-40-76	No

Our purpose here is to present our experience with blood diastase in 17 cases of acute perforated peptic ulcer, all of which came to operation or post-mortem examination. The situation of the ulcer was thus verified in each case.

From Table I it can be seen that in 4 cases of peptic ulcer involving the pancreas, the blood diastase was elevated. In 3 of the 4 cases (Cases 1, 2, and 4) the increase was moderate, while in the fourth (Case 3) it was quite high, coming within the range of figures usually encountered in acute pancreatitis. This case was incorrectly diagnosed acute pancreatitis and operation deferred, although some of the physicians believed it to be a case of perforated ulcer. In spite of the gradual fall in the blood diastase, the patient rapidly became worse and died. Post-mortem examination showed a perforation of a gastric ulcer contiguous to the pancreas. The gradual fall of blood diastase can probably be explained by toxic involvement of the liver. In 13 cases in which the ulcer was situated anteriorly the blood diastase values were within the normal range in 4 cases, and subnormal in 9.

#### DISCUSSION

Meyer and Amtman<sup>3</sup> recently reviewed the meager literature on blood and urinary diastase in peptic ulcers. Their own 3 cases and those of the authors they reviewed were not acutely perforating, but generally of the slowly penetrating type. Since changes in blood diastase occur with great rapidity in acute pancreatic involvement, it is difficult to see how the relatively slow changes taking place in a gradual perforation can effect a constant elevation of the diastase level. We are now studying a group of cases of chronic ulcer with the idea of correlating the diastase levels with the clinical condition of the patients, and possibly with the site of the lesion. It is conceivable that a sudden but small progression of the perforation could be manifested by a sudden rise in the diastase level, while a walling-off of the perforation would result in a rapid reversion to the previous level. Such being the case, the inconclusive results reported in the Meyer and Amtman review are readily understood.

The cases we are presenting—and we are dealing here with ulcers already perforated—seem to indicate that the perforation of a peptic ulcer posteriorly into or near the pancreas causes sufficient pancreatic damage to result in a moderate elevation of the blood diastase level. In those cases perforating away from the pancreas the diastase level is subnormal or at the lower limits of normal. The anterior surface is the most frequent site of perforation. According to Walton,<sup>4</sup> most authors state that about 90 per cent of perforated ulcers are located on the anterior surface. In 59 of his own cases only one perforated posteriorly. Thompson,<sup>5</sup> in a series of 500 cases, noted 69.0 per cent anterior perforations, 27.6 per cent perforations elsewhere, and 3.4 per cent perforations no site recorded. Hence the routine utilization of blood diastase determinations in all cases of acute upper abdominal disorders would seem to aid not only in the differentiation from and recognition of cases of acute pancreatitis, but also in helping to locate the site of perforation of a peptic ulcer if such a lesion exists. Thus, given a case of sudden, sharp, upper abdominal pain and clinical signs suggestive of acute upper abdominal pathology, a low or subnormal diastase level during the height of the attack tends to rule out as the cause either acute pancreatitis or an acute perforation into

the pancreas; a moderately elevated diastase tends to indicate pancreatic involvement from a perforated ulcer; a very high diastase is indicative of acute pancreatitis.

Case 3 offered distinct difficulties. It is true that the initial diastase value of 600 is considered to be within the range of figures generally associated with acute pancreatitis. The suggestive ulcer history in this patient and the clinical picture made most of the physicians who saw its course feel that it was a perforated ulcer. However, the high diastase was permitted to play too prominent a role, and the conservative treatment for acute pancreatitis was instituted. This type of case, however, forms a very small per cent of the perforated ulcers.

No certainty exists as to the nature of the lesion in the pancreas which initiates the elevation of blood diastase. Experimental work on this subject has shown that the conditions which produce rapid elevation of blood diastase values are traumatization of the pancreas and obstruction to the pancreatic duct system. Neither of these conditions was found in Case 3, which at autopsy showed a peptic ulcer perforating onto but not into the pancreas. In this case there was no demonstrable involvement of pancreatic acinar tissue, although the capsule showed inflammatory changes.

Since the changes in urinary diastase follow those in the blood with a lag of several hours,<sup>1</sup> it is obvious that more immediate information can be obtained if determinations are made on blood instead of urine specimens, as others have advocated. This is particularly so if the case is seen within a few hours after perforation.

Finally, it should be emphasized that the diastase estimation is a laboratory procedure, and in no way a substitute for careful clinical judgment. We feel, however, that valuable aid may be given to the differential diagnosis by this test.

#### CONCLUSION

Blood diastase determinations are normal or subnormal in anterior perforated peptic ulcers, and moderately raised in posterior perforated ulcers near or at the pancreas. This helps to differentiate perforated ulcers from acute pancreatitis where the diastase is very high.

#### REFERENCES

1. Somogyi, M.: Methods for the Estimation of Blood Diastase, *J. Biol. Chem.* 125: 369, 1938.
2. Gray, S. H., Probst, J. G., and Heifetz, C. J.: Transient Acute Pancreatitis, *Ann. Surg.* 108: 1029, 1938.
3. Meyer, K. A., and Amtman, L.: Urinary Diastase in Peptic Ulcer Penetrating Into Pancreas, *Am. J. Surg.* 33: 307, 1936.
4. Walton, A. J.: Surgery of Stomach and Duodenum, *Nelson's System of Surgery*, vol. 5, page 47, 1937.
5. Thompson, H. L.: Acute Perforation of Peptic Ulcer, *Surg., Gynec. & Obst.* 64: 863, 1937.

## THE USE OF URETHANE IN SYMPTOMATIC TREATMENT OF BRONCHIAL ASTHMA\*

LAURANCE FARVER, M D NEW YORK, N Y

**I**N EXPERIMENTS on the prevention of anaphylactic shock, Besiedka<sup>1</sup> found that sensitized guinea pigs, which received the shocking reinjection while under ether, urethane, ethyl chloride, or alcohol narcosis, could frequently be saved from fatal shock. Bauzhauf and Famulener<sup>2</sup> later showed that chloral hydrate had the same inhibitory effect. Rosenau and Anderson<sup>3</sup> repeated Besiedka's experiments with ether and urethane narcosis, but were not able to confirm his results. Since then the question whether narcosis especially ether narcosis, can prevent anaphylactic shock has been very controversial, various authors confirming or refuting Besiedka's observations. (See Quill, L M J A M A 109: 854, 1937.)

Some time ago I<sup>4</sup> again took up this problem, and in my experiments administration of urethane to sensitized guinea pigs prior to the shocking reinjection led to the survival of 15 out of 30 animals. In the control experiments only 4 out of 25 guinea pigs survived. Although ether inhalation led to the survival of a greater number of animals than in the controls, the results were not as clear cut as with urethane.

Besiedka suggested that the site of the fatal antigen antibody reaction is in the cells of the central nervous system, and he explained the protective effect of narcotics by the assumption that they "allowed the nerve cell to remain indifferent to the union" of the antigen and antibody. This explanation is distinctly at variance with our present conception that the cause of death of guinea pigs succumbing to acute anaphylactic shock is asphyxia brought about by tetanic contraction of the bronchial muscles.

In *in vitro* experiments I<sup>4</sup> was able to show that urethane inhibits the usual contraction of sensitized plain muscle (uterine strip) which follows contact of the muscle with the antigen. This phenomenon may explain the role urethane plays in the prevention of fatal anaphylactic shock—the tetanic contraction of the bronchial musculature, which leads to asphyxia, is inhibited by the narcotic.

Ethyl urethane ( $C_2H_5OCONH_2$ ) was introduced as a soporific by Selmeideberg<sup>5</sup> in 1886. However, its soporific action is "weak and inconsistent" (Cushny<sup>6</sup>), and as a hypnotic it has been almost entirely replaced by the barbiturates. It forms colorless, odorless crystals which are easily soluble in water. It is given in doses of 1 to 3 gm (Dispensatory of the U S A, 1

\*From the Allergy Clinic, Lenox Hill Hospital, New York.  
Received for publication June 3, 1938.



to 4 gm., Sollmann<sup>8</sup>). The Dispensatory (Centennial, ed. 22) states that "in therapeutic doses it has no perceptible effect upon the respiration or circulation."

I have used ethyl urethane symptomatically as an antispasmodic in 30 cases of bronchial asthma during the last eighteen months. It was administered orally to adults in doses of 1 to 2 gm. (dissolved in water before administration). Not more than 4 gm. per day were given, and it was not used longer than four to five days in succession. No untoward effects nor any narcotic action were observed in any of the patients. In 14 of the 30 cases the alleviating action, which sets in after fifteen to thirty minutes and lasts several hours, was very satisfactory. Some of the benefited patients suffered from severe asthmatic attacks. It is advisable to give urethane before full development of an attack, and in some instances to use 1.0 gm. three times daily over a period of three to four days.

#### SUMMARY

1. In vivo and in vitro experiments in sensitized guinea pigs suggested the use of ethyl urethane in the symptomatic treatment of bronchial asthma.

2. Two to 4 gm. ethyl urethane per day were administered orally in 30 cases of bronchial asthma. The favorable symptomatic action of this drug in 14 of these cases warrants further clinical trials.

#### REFERENCES

1. Besredka, A.: Comment peut-on combattre l'anaphylaxie? *Ann. Inst. Pasteur* 21: 950, 1907.
2. Banzhaf, E., and Famulener, L.: The Influence of Chloral Hydrate on Serum Anaphylaxis, *J. Infect. Dis.* 7: 577, 1910.
3. Cushny, A. R.: *A Text-Book of Pharmacology and Therapeutics*, ed. 11, Philadelphia, 1936, Lea & Febiger, p. 233.
4. Farmer, L.: The Influence of Narcotics on Anaphylactic Shock, *J. Immunol.* 32: 195, 1937.
5. Farmer, L.: The Influence of Urethane on Anaphylactic Reactions, *J. Immunol.* 33: 9, 1937.
6. Rosenau, M. J., and Anderson, J. F.: Further Studies Upon the Phenomenon of Anaphylaxis, *Hyg. Lab. Bull.* 50: 1909.
7. Schmiedeberg, O.: Ueber die pharmakologischen Wirkungen und die therapeutische Anwendung einiger Carbaminsäure-Ester, *Arch. f. exper. Path. u. Pharmacol.* 20: 206, 1886.
8. Sollmann, T.: *Manual of Pharmacology and Its Application to Therapeutics and Toxicology*, ed. 3, Philadelphia, 1927, W. B. Saunders, p. 759.

# COEXISTENCE OF DIABETES MELLITUS AND DIABETES INSIPIDUS\*

## REPORT OF A CASE WITH PREGNANCY

JAMES A. GREENE, M.D., AND R. B. GIBSON, PH.D., IOWA CITY, IOWA

**D**IABETES mellitus and diabetes insipidus rarely coexist in the same patient. A review of the literature reveals that only 20 cases have been reported.<sup>1</sup> In some of these cases the diagnosis of diabetes insipidus was not established according to the present standards; in others, the existence of diabetes mellitus may be questioned. Additional cases of diabetes insipidus described in the literature have had a transient glycosuria or a history of glycosuria. It is difficult, therefore, to estimate the number of reported cases which actually had both diseases.

Recent observations associating diabetes insipidus with lesions of the posterior hypophysis,<sup>2</sup> and diabetes mellitus with dysfunction of the anterior lobe of the pituitary,<sup>3</sup> have aroused added interest in the cases in which these two maladies coexist.

In the presence of diabetes mellitus the incidence of pregnancy is reduced, and, according to Soule,<sup>4</sup> only 37 instances of pregnancy occurring in patients with diabetes insipidus have been recorded in the literature. We have been unable to find a report of pregnancy occurring in a case of coexisting diabetes insipidus and diabetes mellitus. It is for these reasons that we are reporting a case of coexisting diabetes mellitus and diabetes insipidus in which pregnancy developed.

### CASE REPORT

A white woman, aged 25 years, entered the University Hospitals for the first time in May, 1931, complaining of polydipsia, polyphagia, polyuria, fatigability, and loss of body weight. She was found to have diabetes mellitus with glycosuria (graded 4 plus), and a blood sugar of 510 mg. per cent. The diabetes was controlled with a diet which had a potential yield of 109 gm. of glucose, and 29 units of insulin daily. The daily urinary volume varied from 2.5 to 3.8 liters, and the specific gravity of the aglycosuric urine fluctuated from 1.004 to 1.010. Her second admission was in May, 1935, for stomatitis and diabetes mellitus. She stated that for the past five years the menses had occurred only one time each year, were scanty, lasted for only a few hours, and were followed for three days with headache, backache, nausea, and vomiting. Pregnancy had not developed, although contraceptives had not been employed. A glycosuria (graded 4 plus) was present, and the blood sugar was 500 mg. per cent. The glycosuria ceased when the above diet and 42 units of insulin were administered daily. The urinary volumes varied from 2.5 to 3.2 liters per twenty-four hours, and the specific gravity of aglycosuric urine was from 1.004 to 1.010. Her third admission was February 25, 1936, for pregnancy. The diabetes mellitus had been neglected for several days and diabetic coma developed soon after admission. The glycosuria disappeared with a diet which had a potential yield of 153 gm. of glucose, and daily insulin

\*From the Departments of Internal Medicine and Pathological Chemistry, State University of Iowa College of Medicine, Iowa City.

Received for publication, June 6, 1936

dosage of 70 units. The daily aglycosuric urine volumes varied from 6.9 to 8.7 liters, and the specific gravity fluctuated from 1.002 to 1.004. The twenty-four-hour fluid intake was limited to 1.5 liters for three days, and during the last day she excreted only 2.2 liters of urine, but the specific gravity did not increase above 1.005. Thirst became so extreme that the fluid intake had to be increased. Following daily nasal insufflation of dried posterior pituitary gland, the twenty-four-hour urine volume decreased from 8 liters to 4 liters, but the urinary specific gravity was not altered. The administration of posterior pituitary extract parenterally upon two occasions reduced the daily urine volume to 1.8 liters and increased the specific gravity to 1.012. The pregnancy terminated at full term with a normal labor, but the normally developed male infant died two days later from cerebral hemorrhage.

The patient's fourth admission was October 20, 1936, for sterilization. The diabetes mellitus had been controlled with a diet which had a potential yield of 172 gm. of glucose and 55 units of insulin daily. The polyuria and polydipsia of the diabetes insipidus had been controlled with nasal insufflation of dried posterior pituitary gland. The daily urine volume varied from 1.7 to 2.8 liters, and the specific gravity of aglycosuric urine fluctuated from 1.004 to 1.012.

#### COMMENT

Diabetes mellitus and diabetes insipidus may coexist more frequently than is recognized. In a case of known diabetes insipidus, glycosuria is very likely to be discovered, but a persistent polyuria and a low urinary specific gravity are apt to be overlooked if they occur in a patient with diabetes mellitus. The presence of diabetes insipidus was not recognized in our case until the third admission to the hospital, and it was overlooked in the case reported by Gibson, Magers, and Dulaney<sup>11</sup> until the fourth admission.

The presence of diabetes mellitus materially decreases the chance of pregnancy pursuing a normal course. In diabetes insipidus premature labor is frequent<sup>4</sup> and may be induced also by the therapeutic use of extracts of the posterior hypophysis. Gestation continued in our patient to full term. Nasal insufflation of dried posterior pituitary gland diminished the polyuria and polydipsia, but did not produce painful uterine contractions. The first administration of posterior pituitary extract parenterally produced labor pains, but did not induce labor. Whereas, labor was induced by the second injection. The extracts of the posterior hypophysis were administered parenterally to ascertain the effect upon the specific gravity of the urine and to induce labor. The latter is a common procedure in the obstetrical department of this hospital.

The internal secretion of the posterior hypophysis has been thought to be a link in the chain of hormonal control of uterine contractility. Reynolds,<sup>5</sup> however, after a review of the published data, opposes this hypothesis. Furthermore, abnormal labor has not been an outstanding feature in the pregnancy of cases of diabetes insipidus. The labor in our case was normal.

Diabetes mellitus or diabetes insipidus may develop with pregnancy, or if present previously, they may become more severe. Both maladies were aggravated during pregnancy in our patient.

In instances of disturbed carbohydrate metabolism in man, a method is not available by which the frequency of dysfunction of the anterior hypophysis can be ascertained. It has been thought for some time that the same etiologic factors were not present in all cases of diabetes mellitus. Dysfunction of the anterior hypophysis may be the primary etiology in certain instances, and, according to the present concept, there is a hypersecretion in such cases.

Hyperplasia of the anterior lobe with atrophy of the posterior lobe of the pituitary gland is possible in cases of coexisting diabetes insipidus and diabetes mellitus. In our case, however, the roentgenograms of the sella turcica and studies of the visual fields revealed no evidence of enlargement of the pituitary.

## SUMMARY

The coexistence of diabetes insipidus and diabetes mellitus rarely occurs, but may be more common than recognized if the possibility of diabetes insipidus is considered in cases of diabetes mellitus. The occurrence of pregnancy in a case of coexisting diabetes insipidus and diabetes mellitus is reported for the first time.

The relationship of the two maladies to each other and to pregnancy is discussed.

## REFERENCES

- 1 (a) Senator, H. The Relationship Between Diabetes Mellitus and Insipidus. *Deutsche med Wchnschr* 23 385, 1897.
- (b) Brackett, E. J. Acute Diabetes Mellitus Supervening in a Case of Diabetic Insipidus, *Conn. Dentl, Lancet* 2 1433 1899.
- (c) Kuhn, P. Simultaneous Diabetes Insipidus and Mellitus. *Munchen med Wchnschr* 49 103, 1902.
- (d) Heiberg, K. A. A Case of Simultaneous Diabetes Insipidus and Diabetes Mellitus, *Ztschr f klin Med* 73 319, 1911.
- (e) Lewin, C. A Case of Coexisting Diabetes Mellitus and Diabetes Insipidus. *Med Klin* 15 133, 1919.
- (f) Freund, H. Simultaneous Diabetes Mellitus and Diabetes Insipidus. *Klin Wchnschr* 1 1780, 1922.
- (g) Lauter, S., and Hiller, F. Diabetes Mellitus and Diabetes Insipidus Following Pregnancy, *Deutsches Arch f klin Med* 146 335 1925.
- (h) Cipriani, C., Moracchini, R. Acromegaly in Association With Diabetes Mellitus and Insipidus, *Boll Soc ital di biol sper* 2 727 1927.
- (i) di Guglielmo, G. Diabetes Mellitus With Lesion of the Hypophysis. *Boll d Soc med chir di Paris* 2 319, 1927.
- (j) Gibson, R. B., Magers, E. J., and Dalaney, H. Blood Sugar Curves in Diabetes Insipidus and in Habitual and Experimental Excessive Water Drinking, *Endocrinology* 11 341, 1927.
- (k) Cipriani, C., Moracchini, R., and Rotta, C. Diabetes Insipidus and Diabetes Mellitus in Acromegaly With Gorter, Adenoma of Pituitary Gland, and Sclerosis of the Pancreas. *Arch per le sc Med* 53 337, 1929.
- (l) Sweeney, J. S. Coexistent Diabetes Mellitus and Diabetes Insipidus With Case Report, *Endocrinology* 13 477 1929.
- (m) Schuntermann, C. E. Case of Diabetes Insipidus With Diabetes Mellitus, *Klin Wchnschr* 9 22 1930.
- (n) Allan, F. N., and Rowntree, L. G. The Association of Diabetes Insipidus and Diabetes Mellitus. *Endocrinology* 15 97 1931.
- (o) Gellerstedt, N., and Grill, C. A Clinical and Anatomical Contribution to the Knowledge of Combined Diabetes Mellitus and Insipidus, *Acta med Scandinav* 75 586, 1931.
- (p) Duvour, M., Pollet, L., and Cachin, M. Diabetes Insipidus Followed by Diabetes Mellitus With Consideration of Influence of Pregnancy on Polyuria. Results of Folliculin Therapy, *Bull et mem Soc med d hop de Paris* 48 1444 1932.
- (q) Lawrence, R. D., and McCance, R. A. Diabetes Mellitus and Diabetes Insipidus, Associated in One Case, *Lancet* 1 76, 1933.
- (r) Lindeboom, G. A. A Case of Combined Diabetes Insipidus and Mellitus, *Deutsches Arch f klin Med* 175 74, 1933.
- 2 Ingram, W. R., Fisher, C., and Ranson, L. W. Experimental Diabetes Insipidus in the Monkey, *Arch Int Med* 57 1067, 1936.
- 3 Long, C. N. II. The Influence of the Pituitary and Adrenal Glands Upon Pancreatic Diabetes, *Medicine* 16 215, 1937.
- 4 Soule, S. D. Diabetes Insipidus and Pregnancy, *Am J Obst & Gynec* 33 878, 1937.
- 5 Reynolds, S. R. M. The Nature of Uterine Contractility, A Survey of Different Trends, *Physiol Rev* 17 304, 1937.

## THE EFFECT OF SIMULTANEOUS MULTIPLE INJECTIONS OF INSULIN ON THE BLOOD SUGAR\*

EDWARD TOLSTOI, B.A., M.D., AND JANETH RAVNER, M.D.  
NEW YORK, N. Y.

THE speed and duration of action of an insulin preparation is proportional to the rate at which it is absorbed from the injected site. If the absorption is rapid, the pharmacologic effect is reflected quickly by the precipitous fall in the blood sugar; if, on the other hand, the absorption is slow, the blood sugar will drop at a slower rate, and at the same time the duration of action will be prolonged. Furthermore, the rate at which an insulin preparation will be absorbed is dependent upon its relative solubility either before or after it is injected. Regular insulin is an example of a rapidly acting preparation, while protamine insulin is a preparation which acts slowly and over a longer period of time.

Until Hagedorn's introduction of protamine insulin into the treatment of diabetes mellitus in 1936, there was little or no occasion for much deviation from the established technique in the administration of insulin. This was usually given in one, two, three or more doses, twenty to forty minutes before meals, depending of course on the severity of the diabetes, the liberality of the diet, and complications. At no time were two subcutaneous injections administered simultaneously. As the knowledge concerning protamine insulin increased because of the extensive clinical use of the product, it was demonstrated that in a considerable group of diabetic patients, the glycosuria and hyperglycemia could be best controlled by means of two simultaneous insulin injections—protamine insulin into one site and regular into another. This procedure introduced another variable, namely, whether insulin injected into two or more sites acts more swiftly than when the same quantity is administered into a single area. The answer to this question may be of practical value as the multiple method of therapy is used rather extensively in clinical practice. The idea that a difference in the rate of effectiveness of insulin due to one or several sites of injection has occurred to others. It was discussed in connection with the use of protamine insulin by Sprague, Blum, Osterberg, Keppler, and Wilder.<sup>1</sup> They stated that there was no difference in the rapidity of action of the injected insulin whether given into one or several sites. They were careful to state, however, that their observation was confined to a single case. At the time of their publication our experiments were in progress and both because of hypothetical considerations and because our

\*From the New York Hospital and the Department of Medicine and the Department of Pathology, Cornell University Medical College, New York.  
Aided by a grant from Eli Lilly & Co.

Received for publication, June 9, 1938.

results were not in keeping with their conclusions we decided to pursue the problem further. It seemed to us that more insulin—soluble or insoluble—should be absorbed from multiple areas than from a single one and consequently that more sugar should disappear from the blood in the instances where multiple injections had been given. The following experiments were conducted in connection with this problem.

Five dogs were depancreatized. A period of ten to fourteen days was then permitted for recovery from the operation. During this convalescent period blood sugar determinations and analyses were made to establish the diagnosis of diabetes mellitus. Three of the animals revealed a pronounced fasting hyperglycemia, and with these animals a total of six experiments were done. In the remaining two depancreatized dogs, the fasting blood sugars were within normal limits, but the animals did show a postprandial glycosuria as well as an abnormal blood sugar curve after a glucose tolerance test. This curve was of a configuration accepted as diabetic. With these two animals three experiments were performed. The diet was of no particular importance as it related to the experiments. Water was given ad libitum. Before commencing our studies with insulin, blood sugar determinations were done at

TABLE I  
BLOOD SUGAR IN MG PER 100 CC

NO OF UNITS OF INSULIN	FAST ING	2 HR	LOSS PER CENT	4 HR	LOSS PER CENT	6 HR	LOSS PER CENT	FASTING A M FOLLOWING
Control	270	370		285		301		307
1 15 Units R 1 site	377	204	45.0	70	81.5	70	81.5	339
1 15 units R 3 sites	363	51	91.0	37	90.0	59	8.7	310
2 10 Units P 1 site	10	114	64.0	43	86.2	39	8.5	247
2 20 Units P 3 sites	207	33	89.3	20.2	93.5	18.6	94.0	195
3 30 Units P 1 site	345	257	25.5	154	55.0	109	68.4	190
3 30 Units P 3 sites	198	132	33.5	71	64.2	50	75.0	142
4 40 Units P 1 site	400	270	32.5	210	47.5	176	56.0	250
4 40 Units P 3 sites	312	205	35.0	107	65.7	50	84.0	
5 15 Units P 1 site	301	141	53.0	64	79.0	87	71.0	
5 15 Units P 3 sites	328	72	78.0	58	82.9	91	72.2	177
6 20 Units P 1 site	255	178	30.0	173	32.5	128	50.0	66
6 20 Units P 3 sites	209	286	17.0	165	59.0	100	68.6	109

P = Protamine insulin

R = Aqueous soluble insulin

two-hour intervals on one animal in each group. This served as a control and demonstrated that there was no constant diminution of the sugar concentration in the blood when no insulin was given. After the controls were established, we proceeded as follows: Insulin was withheld from all the animals for at least a four-day period. Then on the day of the experiment the fasting blood sugar level was established and a single dose of insulin—either regular or protamine—was given hypodermically. After the administration of insulin, the blood sugar level was determined at two-hour periods. Two or three days later a *similar* dose of insulin was divided into three portions and given to the same animal into three different sites, the blood sugar determinations being carried out at the same time intervals as in the former experiment. The blood was obtained from the femoral artery and prevented from clotting by the use of potassium oxalate, approximately 2 mg. per c.c., and the Folin and Wu method was employed for the blood sugar analyses which were done as soon as the blood was drawn so that the question of a glycolysis might not enter into the evaluation of the data. The results are presented in the tables

TABLE II  
BLOOD SUGAR IN MG. PER 100 C.C.

	NO. OF UNITS OF INSULIN	FAST- ING	2 HR.	LOSS PER CENT	4 HR.	LOSS PER CENT	6 HR.	LOSS PER CENT	FASTING AM FOLLOWING
	Control	113	105		97		105		103
1.	15 Units R. 1 site	119	35	70.3	48	60.0	58	51.5	122
	15 Units R. 3 sites	99	42	58.0	38	42.0	57	43.0	102
2.	30 Units P. 1 site	107	72	32.5	51	52.5	46	57.0	66
	30 Units P. 3 sites	90	52	42.0	47	48.0	48	46.5	98
3.	20 Units P. 1 site	102	70	31.5	62	39.0	50	51.0	106
	20 Units P. 3 sites	87	53	39.5	38	56.5	21	76.0	90

P = Protamine insulin

R = Aqueous soluble insulin.

The data presented in Table I show that in most of the experiments, the percentile loss of glucose from the blood is much greater when insulin, regular or protamine, is given into multiple sites. This is particularly true in the presence of a definite hyperglycemia at the beginning of the experiment. If however, the experiments were done at times when the blood sugar was normal, the results were not quite as striking nor as consistent. In this latter group one experiment was done in which regular insulin was used, and two similar experiments were done with protamine insulin. Table II reveals that in only one of the three experiments was the loss of blood glucose greater when the insulin was given into three sites. The explanation for this difference in results may be given by the hypothesis that with a normal blood sugar

the regulatory factors are probably called into play, thus maintaining such a biologic constant as the blood sugar within certain definite limits. This group of experiments is only of academic interest as in practice insulin is not often used when the blood sugar is normal. Our results however in the hyperglycemic experiments may prove of value in clinical practice and furthermore, may account for the unexpected reactions in instances where the multiple technique of injection is employed.

#### SUMMARY

Five diabetic (depancreatized) dogs were used as experimental subjects to determine how a similar quantity of insulin administered into one or three areas, would influence the blood sugar curve. In the hyperglycemic animals the blood sugars fell more rapidly when a given quantity of insulin was administered into three sites than when a similar amount was given into one area.

#### REFERENCES

- 1 Sprague, R. G., Blum, B. B., Osterberg, A. E., Keppler, E. J. and Wilder, R. M. Clinical Observations With Insulin Protamine Compounds. *J. A. M. A.* 106: 1701, 1936.
- 2 Hagedorn, H. C., Jensen, B. N., Krarup, N. B. and Wodstrup, L. Protamine Insulinate, *J. A. M. A.* 106: 177, 1936.

---

## A COMPARATIVE STUDY OF MEDIA EMPLOYED IN THE ISOLATION OF TYPHOID BACILLI FROM FECES AND URINES\*

CORA B. GUNTHER, M.S. AND LOUIS TUFT, M.D. PHILADELPHIA, PA.

---

A PERFECT medium for the isolation of typhoid bacilli from feces and urines is one which will allow the typhoid organisms to grow luxuriantly and entirely inhibit the coli-aerobacter group. Moreover, it should be easy to read, easily prepared, stable and uniform, so that it will give consistent results in the hands of most workers. Such an ideal medium has not yet been produced. In an attempt to devise this perfect medium, a fascinating and colorful array of enrichment and plating media has been tried and highly recommended by their originators.

A glance at the list of media advised by the better known laboratory manuals and textbooks shows that there is ample choice. The eighth edition of "Stitt"<sup>1</sup> lists seven media, while the 1927 edition of Ford's<sup>2</sup> *Bacteriology* mentions twelve media for the isolation of intestinal pathogens. The newer editions of textbooks generally limit their recommendations to a few of the most widely used or to the newer ones which have proved superior to many of the older ones. Kolmer and Boerner (1937)<sup>3</sup> list only three of the newer media: selenite F enrichment, Leifson's desoxycholate citrate, and Wilson-Blair's bisulphite sulfite. Zinsser and Brune Jones<sup>4</sup> suggest Endo, Krumweide's

\*From the Pennsylvania State Health Laboratories, Philadelphia.

Read before Eastern Chapter of the Society of American Bacteriologists, April 26, 1938.  
Received for publication, June 11, 1938.



brilliant green, and eosin-methylene blue as the three most serviceable. These three media, together with MacConkey's bile salt medium and Teague's 30 per cent glycerin enrichment, are perhaps the most widely used.

All of the various media in use take advantage of some cultural or biologic characteristic of the typhoid bacillus to differentiate it from the other gram-negative intestinal rods. A closer scrutiny of these differential media shows that they can be divided roughly into two groups (1) differential only and (2) differential and inhibitive.

Many media belonging in the first group, the differential group, base their usefulness on lactose fermentation with a suitable indicator, such as litmus, bromthymol blue, etc. A change of indicator is often sufficient reason for launching a new medium. Endo, lactose litmus agar, and eosin-methylene blue media fall into this group. Many of the lactose media have no inhibiting substance against the coli-aerobacter group. Under these conditions, the coli group has the advantage. Wilson<sup>5</sup> states the case very aptly when he says: "With media depending for the differentiation between colonies of *B. typhosus* and *B. coli* on the presence of lactose, the dice are loaded against *B. typhosus*, since an extra supply of energy in a utilizable form is being supplied to the *B. coli*."

The second group of media, besides being differential, is also selective or inhibitive, i.e., these media contain some agent such as brilliant green or bile salts which inhibits the coli-aerobacter group. The disadvantage of these highly selective media is that they must be very carefully balanced in order to inhibit the proper organism. This difficulty, however, can be overcome. Within the past few years a number of highly selective media have been developed which have given very encouraging results in the hands of their originators.

With these points in mind, we decided to test the claims of several of the newer media by comparing them with the older and more familiar ones. The newer media used in this study are: Leifson's selenite F enrichment, desoxycholate plating media, and Wilson-Blair's bismuth sulfite plating medium.

In 1935<sup>6</sup> and 1936,<sup>7</sup> Leifson described a combination of selenite F enrichment and desoxycholate citrate plating media.\* The selenite F enrichment medium contains 0.4 per cent sodium acid selenite, lactose, and sodium phosphate. This concentration of the selenite salt favors the growth of typhoid and paratyphoid bacilli and inhibits the coli group. Leifson claims that, in some cases, the typhoid bacilli multiply a million times. A 10-20 per cent emulsion of feces is made and incubated twenty-four hours at 37° C. before streaking on desoxycholate citrate or other plating medium.

Leifson's desoxycholate citrate medium contains sodium desoxycholate, lactose, ferric sodium citrate, and sodium citrate with neutral red indicator. The medium is pale reddish yellow before inoculation. The typhoid bacillus appears yellow while the coli-aerobacter group turns dark pink or red, due to the precipitation of desoxycholic acid. The citrate, however, inhibits many of the coli group. Undiluted feces can be streaked directly on the medium. the incubation period being twenty-four hours.

\*These media have been purchased by us from the Baltimore Biological Co., Baltimore

The Wilson Blair bismuth sulfite medium is not a new medium in point of time, but rather in point of use. Wilson<sup>8</sup> published the first work on it in 1923 and since then it has gone through many modifications. It has been rather widely used in European countries, but attracted very little attention in this country until about two years ago when the Difco Co. modified it and put it on the market in a dehydrated form. Since then it has been gaining in popularity. Prior to that time the difficulty of preparation deterred many laboratories from using it.

The Wilson Blair medium is a pale greenish gray opaque medium. On this medium, the typhoid bacillus produces a conspicuous, flat, black lustered colony with a smoky halo. Wilson<sup>9</sup> explains the blackening of the colonies thus: "(1) *B. typhosus* in the presence of a fermentable carbohydrate is able to reduce sulphite to sulphide and form a black colony in the presence of an iron salt. (2) Bismuth sulphite in the presence of an excess of sodium sulphite suppresses most coliform bacilli. Brilliant green intensifies the selective action." The blackening of the colonies however requires a very delicate balance between the constituents. Table I shows the constituents of the medium in various modifications, with the function of each briefly stated. It can be seen that the Difco product corresponds closely to the Tabet<sup>10</sup> modification, which is more sensitive than Wilson's 1933<sup>11</sup> formula.

TABLE I  
COMPOSITION OF WILSON MEDIUM IN VARIOUS MODIFICATIONS

CONSTITUENTS	WILSON 1933	TABET 1938	DIFCO 1936	FUNCTION
Bismuth ammonium citrate (scales)	0.6*	0.6	0.8 Bis muth sulphite indi cator	Indicator of hydrogen sulfide production
Anhydrous sodium sulfite	2.0	1.0		Suppression of <i>B. coli</i> , excess inhibits <i>B. paratyphosus B</i>
Anhydrous sodium phosphate ( $\text{Na HPO}_4$ )	1.0	0.35	0.4	Buffer excess inhibits typhoid bacillus
Glucose	1.0	1.0	0.5	Supplies energy
Ferrous sulfate	0.08	0.08	0.03	Counteracts inhibition of bis muth sulfite
Brilliant green	0.005	0.005	0.0025	Suppression of <i>B. coli</i>

\*Grams per 100 c.c.

We have used the Difco medium in our comparative experiments and have found it highly satisfactory. This product removes the objections to the use of Wilson's original medium, i.e., difficulty of preparation and instability. The medium is extremely simple to prepare. Fifty-two grams of the powder is dissolved in 1 liter of water, boiled for a few minutes and poured. Plates are allowed to stand open until thoroughly dry. Workers using the Wilson formula state that the medium is not good for more than four or five days after its preparation. We find that the Difco plates kept in the icebox for two weeks still produce characteristic black colonies.

The medium is streaked directly with undiluted feces incubated forty-eight hours, and read. Most of the coli group are inhibited. The typhoid

bacilli produce such characteristic black colonies that they can easily be detected even if there are many other colonies on the plate. The only non-pathogen which could be confused with the typhoid bacillus is a reducing type of *B. coli* which sometimes appears on the plates. These colonies simulate typhoid bacilli, but can easily be ruled out by transfer to Russell double-sugar slants. Besides the blackening of the typhoid colony itself, the medium under the colony is blackened. If the colony is scraped off with a loop, a black area will be found in the medium. The typhoid colony is not the only one which can reduce the medium; if an organism shows a characteristic surface colony and also reduces the medium beneath it, this is additional evidence that you may be dealing with a typhoid bacillus. All reducing colonies such as *B. paratyphosus* B, *S. enteritidis*, and reducing *coli* produce this blackening. The only other point to be kept in mind in reading the plates is the fact that if the plate is crowded with typhoid bacilli they may not all blacken. A few at the edge may blacken and the rest remain green.

Since the blackening of the colony depends on hydrogen sulfide production, a question naturally arises as to whether all typhoid colonies blacken uniformly. We have encountered only one freshly isolated strain obtained from a typhoid release patient, which consistently failed to blacken and which produced a green colony. We have called this a "green typhoid" for want of any other descriptive term. Four subsequent specimens obtained from the same patient showed exactly the same colony on each occasion. The organisms obtained from these colonies correspond both culturally and serologically to the typhoid bacillus. A subculture examined by Colonel Hitchens of the Army Medical School was reported as serologically identical with and as virulent as the Panama carrier strain No. 58. We inquired of other workers who had been using Wilson medium extensively and found that none of them had encountered similar strains. It would appear, therefore, that such atypical strains, fortunately, are very rare and would not militate against the use of this medium.

Highly selective media for the typhoid bacillus often have a limited usefulness for other enteric organisms. To find out whether Leifson's and Wilson's media have as wide an application as some of the less selective media, we streaked a number of stock cultures on them. Table II shows the results of these tests. Failure of old stock cultures of the typhoid bacillus to grow on Wilson medium has been noted by other workers and is attributable to the age of the culture. We have never, however, encountered a freshly isolated strain that failed to grow on the medium. In general, both media are good for typhoid bacilli and some salmonellas. Their use for the dysentery group is limited. None of the pathogenic dysentery strains grow on Wilson's medium. Flexner strains and Schmitz bacillus are apparently the only ones which grow consistently on the Leifson medium. The fact that most of the stock strains of the *escherichia-aerobacter* group grew well on both media does not prove that the media are favorable to these organisms. With a heavy inoculation some organisms will grow.

TABLE II  
CULTURAL CHARACTERISTICS OF ENTERIC ORGANISMS

NO TEST ED	ORGANISM	LEIFSON'S MEDIUM		WILSON'S MEDIUM	
		NO GROWN	DESCRIPTION OF COLONY	NO GROWN	DESCRIPTION OF COLONY
10	<i>B. typhosus</i>	9	Clear yellow	9	Flat black with metallic
13	<i>B. dysenteriae</i> , Flexner, Hiss, Sonne	11	Yellow opaque	0	luster and halo
4	<i>B. dysenteriae</i> , metadysenteroides, dispersalcalescens	3	Whitish opaque	4	Green black variable
5	<i>B. paratyphosus</i> A	5	Yellow translucent	5	Green variable
12	<i>B. paratyphosus</i> B	12	Yellow opaque	12	Moist black with luster and halo, at times variable
1	<i>S. aertrycke</i>	1	Yellow	1	Flat green
1	<i>S. enteritidis</i>	1	Yellow	1	Similar to <i>B. paratyphosus</i> B
1	<i>S. sursestifer</i>	1	Yellow	1	Flat green
4	<i>S. morganii</i>	4	Yellow	4	Flat green
1	<i>S. columbensis</i>	1	Yellowish opaque	1	Dark green mucoid
4	<i>B. proteus</i>	3	Yellowish opaque	3	Flat green turning black
7	<i>Escherichia</i>	7	Pink mucoid with milky halo	7	Variable color, mucoid or flat
4	<i>Aerobacter</i>	3	Pink mucoid, without halo	4	Gray mucoid variable

We then carried out a comparative study between Wilson's medium and some of the other commonly used media to determine their relative efficiency in actual typhoid isolation work. Material for this study consisted of feces and urines sent by mail to the Pennsylvania State Laboratory for routine examination for typhoid bacilli. They were taken either from food and milk handlers to rule out carriers, from suspected carriers, from patients in whom typhoid was suspected or where release from quarantine was desired. The specimens were received in small sterile bottles with no preservative. Two

TABLE III  
COMPARISON OF WILSON WITH OTHER PLATING MEDIA

GROUP NO	TECHNIQUE	TOTAL NO EXAM- INED	TOTAL NO POSITIVE	NO POSITIVE ON BOTH	NO POSITIVE ON ENDO ONLY	NO POSITIVE ON WILSON ONLY	ADDITIONAL POSITIVE ON WILSON	
							NO	PER CENT
I	Feces direct on Wilson glycerin suspension on Endo	871	156	80	8	68	60	38.4
II	Glycerin suspension on Endo and Wilson	275	59	41	0	18	18	30.5
III	Paratyphoid B release as in I	8	6	3	0	3	3	50.0
IV	Urine broth culture on Endo and Wilson	197	12	8	0	4	4	33.3
V	Feces direct on Leifson and Wilson	100	20	4	Leifson only 0	16	16	80.0
VI	Feces direct on Wilson, glycerin suspension on eosin methylene blue	100	21	13	Eosin methylene blue only 0	8	8	38.0

thousand two hundred and forty-five specimens of feces and urines were cultured in various combinations.

Since for many years we had depended on enrichment in 30 per cent glycerin and streaking on Endo plates for routine typhoid isolation work, we decided to compare this technique with one using the Wilson medium. The results of these tests are shown in the first four groups of Table III.

In the first of these groups were 871 feces which were streaked directly on Wilson plates and also emulsified in 30 per cent glycerin and streaked on Endo. Fifty-five per cent of these were streaked on one Wilson plate only, and 45 per cent on two separate Wilson plates. A 10-20 per cent suspension of feces was made in the glycerin. A loopful was streaked on an Endo plate before and after incubation at room temperature for twenty-four hours. Taking the total number of positives as 100 per cent, we figured the percentage of additional positives obtained by the use of Wilson medium. In spite of a possible advantage of enrichment in glycerin and two Endo plates compared to one Wilson (in 55 per cent) we got 38.4 per cent more positives by using the Wilson medium.

In group II a 10-20 per cent glycerin suspension was made on 275 feces and a loopful streaked on one Wilson plate and a loopful on Endo before and after incubation. Here again the Endo medium had the advantage of more streakings, but we found 30.5 per cent more positives by the Wilson medium.

Group III contained 8 specimens from a *B. paratyphosus* B release case in which of the 6 positives, 3 additional were detected by means of the Wilson medium.

In group IV were 197 urines. Two cubic centimeters of urine were placed in a veal broth tube and incubated at 37° C. for twenty-four hours. A loopful was streaked on a plate of each medium. We found 33.3 per cent more positives on Wilson medium.

The superiority of Wilson medium over Endo is apparent from these results. Where a strict comparison is made, as when streaking from the same liquid medium, such as a suspension or broth culture, there is still a 30 per cent advantage.

We then compared Wilson's medium with Leifson's and eosin-methylene blue media.

In the series of 100 feces comparing Wilson's with Leifson's medium two plates of each medium were streaked directly with a loopful of feces. We got so many more positives by the Wilson medium that we did not think it worth while to compare more than 100 specimens. Leifson's medium does not inhibit so many *B. coli* as Wilson's medium, and it is much more difficult to read. If there are many *B. coli* or aerobacters on the plate, they turn yellow very quickly. The typhoid bacilli being yellow also makes the plate difficult to read.

In comparing eosin-methylene blue medium with Wilson, a loopful was streaked from a glycerin suspension on to each medium. It can be seen from the chart that eosin-methylene blue medium runs parallel with the Endo in percentage of positive isolations. Therefore, we did not think it worth while to continue any more comparative tests with this medium.

TABLE IV  
RESULTS OF VARYING TECHNIQUE WITH WILSON MEDIUM

GROUP NO	TECHNIQUE	TOTAL NO EXAMINED	TOTAL NO POSITIVE	NO POSITIVE ON BOTH	NO POSITIVE SUSPENSION ONLY	NO POSITIVE DIRECT ONLY	ADDITIONAL POSITIVE DIRECT	
							NO	PER CENT
I	Feces direct on Wilson glycerin suspension on Wilson	275	76	59	1	16	15	19.7
II	Feces direct on Wilson deep Wilson plates	100	21	13	Deep only 1	7	6	28.5
III	Two Wilson plates compared to one Wilson plate	1,289	230	170	One only 60	Additional positive possible by using two plates		
							30	13.0

So far we have demonstrated the superiority of Wilson medium over Endo, eosin methylene blue, and Leifson's media. We then tried to find out the best way of using Wilson medium. Table IV shows the results of varying the technique with Wilson medium.

In group I were 275 feces. Two plates were streaked directly on Wilson and a loopful streaked on one Wilson plate from glycerin suspension. We obtained 19.7 per cent more positives by direct streaking.

There were 100 feces in group II. Two plates were streaked directly on Wilson medium and one deep plate made. The technique for the deep plates consisted in first emulsifying in glycerin, then by means of a 10 c.c. pipette with a wide opening at the bottom, the cotton plug is pushed slowly to the bottom of the tube, carrying with it the large pieces of feces and leaving the glycerin above the cotton comparatively clear. Five cubic centimeters of this filtered emulsion is put into a Petri dish and about 20 c.c. of melted Wilson medium, cooled to 45° C, poured on it. The plate is rotated to mix thoroughly and allowed to stand until thoroughly dry. This plate is incubated for forty-eight hours and read. The typhoid colonies grow in distinct round black colonies right below the surface of the agar. Even with this larger inoculum, we obtained 28.5 per cent more positives by direct Wilson plates.

In group III we compared the efficiency of streaking two Wilson plates with one. Of the total number of 230 positives, 60 were positive on one plate only. One half of these, 30, or 13 per cent, might have been missed had only one plate been used. It can be seen from these figures that Wilson medium can be used more advantageously by streaking two plates direct with undiluted feces. This fact is of distinct advantage in the examination of large numbers of specimens, for example those sent in to public health laboratories from food handlers. In these instances, one Wilson plate could be employed and would still be more efficient than several Endos. The economy of time and materials afforded by this method in laboratories where large numbers of specimens are handled constantly, is obvious.

After demonstrating Wilson medium to be the best medium for streaking, we turned our attention to increasing our isolations, if possible, by preliminary enrichment. Although Leifson recommended the use of selenite F enrichment with desoxycholate streaking medium, we demonstrated in Table III that the

TABLE V  
COMPARISON OF ENRICHMENT MEDIA

GROUP NO.	TECHNIQUE	TOTAL NO. EXAMINED	TOTAL NO. POSITIVE	NO. POSITIVE ON BOTH	NO. POSITIVE WILSON DIRECT ONLY	NO. POSITIVE Selenite F + WILSON ONLY	ADDITIONAL POSITIVES WILSON DIRECT	
							NO.	PER CENT
I	Feces direct on Wilson selenite F on Wilson	670	129	74	33 (25.5%)	22 (17.0%)	11	8.5
II	Urine broth culture and double strength selenite F on Wilson	200	11	3	Selenite F only 0	Broth only 8	Additional positive broth 8	72.7
III	Before and after selenite F on Wilson	275	33	12	Before only 4 (12.1%)	After only 17 (51.5%)	Additional positive selenite F 13	39.3
IV	Before and after glycerin on Endo	496	60	54	Before only 4 (6.6%)	After only 2 (3.3%)		

advantage of the combination was not in the plating medium. Any advantage must, therefore, be present in the enrichment medium. We, therefore, tried a series of enrichment in selenite and then streaking on Wilson compared to direct streaking on Wilson. Table V shows the results of these experiments.

In group I we streaked two Wilson plates directly with undiluted feces and also made an emulsion in selenite F. After incubating the emulsion at 37° C. for twenty-four hours, a loopful was streaked on one Wilson plate. It was found that of the total positives, 25.5 per cent were positive by direct streak on Wilson medium but were negative after preliminary enrichment in selenite and then streaking on Wilson medium. On the other hand, of the same group, 17 per cent were negative by direct streaking on Wilson but were positive after preliminary enrichment in selenite. There was only a small advantage, 8.5 per cent, of the direct streaking over enrichment and streaking. Since we obtained almost the same number of additional positives with each method, we continued using the combined technique of streaking directly, on the one hand, and enriching and then streaking, on the other hand. Our present technique consists, therefore, of direct streaking of undiluted feces on two separate Wilson plates and in addition emulsification of the feces in selenite F and then streaking it on one Wilson plate after twenty-four hours' enrichment.

In group II we compared 2 c.c. of urine in broth and 10 c.c. of urine in double strength selenite F. Both were incubated twenty-four hours and streaked on Wilson medium. We obtained 72.7 per cent more positives with broth cultures.

We further attempted to demonstrate that the selenite enrichment really causes the typhoid bacilli to multiply. One loopful from an emulsion was streaked on Wilson medium before and after incubation. The figures show that this actually happens, as Leifson claims. We could not, however, demonstrate any actual multiplication of typhoid organisms in the glycerin enrichment. Teague does not claim that the organisms multiply but remain the same for a period of a week.

TABLE VI

SUMMARY OF LITERATURE COMPARING WILSON BLAIR MEDIUM WITH OTHERS

MEDIUM	AUTHOR	TOTAL NO EXAMINED	ADDITIONAL POSITIVES ON WILSON PER CENT
Endo	Sellers <sup>12</sup> (1934)	22	44.4
	Shaughnessy <sup>13</sup> (1936)	10 199	50.2
	Gunther and Tuft (1938)	871	38.4
Malachite green	Joost <sup>14</sup> (1934)	10 142	64.5
MacConkey's	Wilson and Blair <sup>5</sup> (1931)	49	50.0
Eosin methylene blue	Gunther and Tuft (1938)	100	78.0
Leifson's	Gunther and Tuft (1938)	100	80.0
Desoxycholate citrate	Sellers <sup>12</sup> (1937)	100 positives	Desoxycholate 10.0
<i>B. paratyphus</i> I			
Endo	Gunther and Tuft (1938)	8	50.0
MacConkey's	Wilson and Blair <sup>5</sup> (1931)	22	28.3
Brilliant green eosin	Glass and Tabet <sup>16</sup> (1938)	212	Brilliant green eosin 4.7
<i>S. enteritidis</i>			
MacConkey's	Wilson and Blair (1931)	14	50.0

In order to see whether our findings with the Wilson medium were corroborated by others we have searched the literature for similar work. Table VI is a tabulation of results obtained by different workers comparing Wilson medium with other media. It can be readily seen that other workers have had the same favorable results with Wilson medium. In the one case in which Sellers (1937) got 10 per cent more positive cultures on Leifson than on Wilson medium, the comparison made is not similar to that made in the present study, since Sellers compared enrichment in selenite and streaking on desoxycholate with direct streaking on Wilson medium. Those streaked on desoxycholate had the advantage of enrichment. Furthermore, those streaked on Wilson medium were emulsified in glycerin. We have shown that the most advantageous way to use the Wilson medium is by direct streaking and not by streaking from an emulsion. In the work of Glass and Tabet<sup>16</sup> comparing brilliant green eosin with Wilson medium for isolation of *B. paratyphus* B, the authors state that the advantage of brilliant green eosin is so small that it is probably due to sampling error. Furthermore the brilliant green eosin is so difficult to read that this small advantage is more than outweighed by the ease of reading the Wilson plates. With the two exceptions we have already discussed, this summary of results obtained in experiments comparing Wilson with other media shows the superiority of the Wilson medium in actual isolation work.

Considering this survey as a whole, the most important practical advantage resulting from it has been our change in technique for typhoid isolation work. This has been changed completely from the glycerin enrichment Endo technique to the selenite enrichment Wilson method, thus increasing our isolations almost 50 per cent. Considering the number of positives obtained with the glycerin Endo technique, we had 88 out of 871 specimens or 10.1 per cent. With the selenite enrichment Wilson technique which we are now using, we



had 129 positives out of 670 specimens, or 19.2 per cent, an increase of almost 50 per cent in efficiency. The importance of this increase in sensitivity in relation to public health work is obvious.

#### SUMMARY AND CONCLUSIONS

1. A total of 2,245 specimens of feces and urines were cultured for typhoid bacilli, employing various media in a comparative way.

2. Wilson-Blair bismuth sulfite medium was found to produce 38.4 per cent more positives than Endo; 38 per cent more than cosin-methylene blue and 80 per cent more than Leifson's desoxycholate medium.

3. A tabulation showing the constituents and mechanism of Wilson-Blair medium in several modifications was presented. The Difco modification used in these tests was found to be superior to Wilson's original formula, being more easily prepared, relatively more stable, and more sensitive. It is useful for the isolation of many salmonellas but not for the dysentery group. The medium is especially adapted for work in public health laboratories where large numbers of specimens from food handlers are examined. Practical points about its preparation and use were stressed.

4. Selenite F enrichment medium was shown to be superior to 30 per cent glycerin water. Typhoid bacilli multiply in the former but not in the latter.

5. From our experience in the use of the Difco modification of the Wilson-Blair medium over a period of two years and the results of our comparative studies, we believe the Wilson-Blair medium to be superior to any other plating medium so far devised for the isolation of typhoid bacilli from feces and urines. We feel that it fills a long-standing need for a satisfactory plating medium for isolation work.

6. As a result of these studies, the technique which we now employ consists in direct streaking on two Wilson plates as well as enrichment in selenite F, with subsequent streaking on Wilson medium. This technique has proved to be almost 50 per cent more sensitive than our previous methods, an advantage which is obvious from the public health standpoint.

#### REFERENCES

1. Stitt, E. R.: *Practical Bacteriology, Blood Work and Animal Parasitology*, ed. 5. Philadelphia, 1927, P. Blakiston's Son & Co.
2. Ford, William W.: *Text-book of Bacteriology*, Philadelphia, 1927, W. B. Saunders Co.
3. Kolmer, John A., and Boerner, Fred: *Approved Laboratory Technic*, ed. 2, New York, 1937, D. Appleton-Century Co.
4. Zinsser, Hans, and Bayne-Jones, Stanhope: *A Text-book of Bacteriology*, ed. 7, New York, 1935, D. Appleton-Century Co.
5. Wilson, W. J., and Blair, E. M. M.: Further Experience of the Bismuth Sulphite Media in the Isolation of the *Bacillus typhosus* and *B. paratyphosus* B From Faeces, Sewage and Water, *J. Hyg.* 31: 138, 1931.
6. Leifson, E.: New Culture Media Based on Sodium Desoxycholate for the Isolation of Intestinal Pathogens and for the Enumeration of Colon Bacilli in Milk and Water, *J. Path. & Bact.* 40: 581, 1935.
7. Leifson, E.: New Selenite Enrichment Media for the Isolation of Typhoid Bacilli, *Am. J. Hyg.* 24: 423, 1936.
8. Wilson, W. J.: Reduction of Sulphites by Certain Bacteria in Media Containing a Fermentable Carbohydrate and Metallic Salts, *J. Hygiene* 21: 392, 1923.
9. Wilson, W. J.: Isolation of *B. typhosus* From Sewage and Shellfish, *Brit. M. J.* 1: 1061, 1928.

- 10 Tabet, F A Modification of Wilson and Blair's Bismuth Medium Suitable for Both Typhoid and Paratyphoid Bacilli J Path & Bact 46 181, 1938
- 11 Wilson, W J Isolation of Enteric Bacilli From Sewage and Water and Its Bearing on Epidemiology, Brit M J 2 560 1933
- 12 Sellers, T F, Morris, Janie F, and Reynolds, Midge Notes on the Application of Bismuth Sulphate Medium to the Isolation of B typhosus From Feces, J LAB & CLIN MED 20 202, 1934
- 13 Shaughnessy, H J, Hall, N, Alberts, J C and Frewer F Experiences With Special Methods in the Isolation and Identification of Typhoid Bacilli From Blood, Feces and Urine, J Bact (Abst) 32 128, 1936
- 14 Joos, I Wilson and Blair's Dextrose Bismuth Citrate Iron Brilliant Green Culture Medium, Zentralbl f Bakt (Abst 1) 132 252, 1934
- 15 Sellers, T F Private communication
- 16 Glass, V, and Tabet, F A Comparison of Brilliant Green Eosin and Bismuth Agar in the Isolation of Bact paratyphosum B from Feces, J Path & Bact 46 195, 1938

---

## THE EFFECT OF LYMPHOCYTES IN VITRO UPON BACTERIAL TOXINS\*

H D MOOR, M D, AND NORSUDA M NEWPORT, M D, OKLAHOMA CITY, OKLA

---

### INTRODUCTION

WITHIN the past thirty five years there have been published a number of articles dealing with the functions of the lymphoid tissue and of the lymphocyte. Speculations and observations by several writers have led to numerous and varied theories as to the role or roles played by the lymphocyte in particular. Until recent years very little experimental work, tending to give a substantial basis for theorizing, has been carried out.

The present studies and experiments have covered a period of two years and a half. During this time a fairly comprehensive review of the available literature was undertaken, and a number of experimental studies were carried out in an effort to substantiate certain theories gained from the literature.

Aside from the blood vascular system there is no system that compares in extent with the vast network of lymphatic capillaries, vessels, ducts, and milions of specialized aggregations of lymphoid tissue along the course of this system. The importance of lymphoid tissue in the general economy of the body is shown by its great abundance, and the importance of its function is indicated by its structure and anatomic relationships.

Time and space do not allow a complete review of the literature bearing upon the function of the lymphocyte in its free and aggregated states. Many interesting features and trends are brought out by various writers such as Vuchon,<sup>1</sup> Boyd,<sup>2</sup> Malloy,<sup>3</sup> Gulland,<sup>4</sup> and many others. There is in none of these reports convincing evidence which places its role or roles upon a substantial foundation. Symposiums of all of the available facts have been published, but none of these are recent. The recent literature is more concerned with the possible significance of the lymphocyte in relationship to malignant

---

\*From the Department of Bacteriology, University of Oklahoma School of Medicine, Oklahoma City.

Received for publication June 14 1938

disease and experimental tuberculosis. The chronic lymphocythemias are becoming fairly well recognized as neoplastic processes.

#### THE EFFECT OF LYMPHOCYTES IN VITRO UPON BACTERIAL TOXINS

Vitro studies, although occupying a prominent place in experimental medicine, have not, as far as the writer can ascertain, gone into this problem heretofore. It is a well-known fact that brain tissue has a very marked affinity for tetanus toxin and vice versa. Outside of this, the work of Pappenheimer<sup>5</sup> (1917) and the observations of Alexeieff<sup>6</sup> (1925), nothing has approached an experimental proof of the alleged deleterious effects of lymphocytes upon toxins. If they do exert this influence, it would be difficult to explain the mechanism. We may suppose that the mechanism is through one of the following phenomena: (1) production of antibodies, (2) enzymatic destruction, (3) surface affixation, (4) precipitation, or (5) phagocytosis. The weight of the evidence seems to point to the latter mechanism, although it is difficult for many to conceive of a phagocytosis of fluids. It must not be forgotten that biological chemists have shown that protein molecules can sometimes have enormous molecular weights and in themselves constitute a very complex, bulky mass of living or dead material.

*Materials and Methods.*—Guinea pigs are most ideally adapted to lethal dose experiments because of their extreme susceptibility to various agents. They are, however, quite expensive. The first experiments were conducted with guinea pigs, but these animals were replaced by white mice because of the cost of the former. It was soon found, however, that mice are certainly not susceptible to certain toxins, which fact also was noted by Metchnikoff<sup>7</sup> (1907); "Mice and rats tolerate large quantities of diphtheria toxin. The rat resists a dose of diphtheria toxin capable of killing several rabbits. This is a natural immunity." The writers injected 28 white mice with diphtheria toxin in doses grading from 0.0005 c.c. to 0.50 c.c. before they ran across this statement of Metchnikoff's. All mice were apparently unaffected, including the one receiving 0.50 c.c. of undiluted toxin, fifteen times enough to kill a 704 gm. guinea pig in forty-one hours. Mice were also found to be little or not at all affected by tuberculin and typhoid bacillus extracts. The former was the commercial "old tuberculin," and the latter was made by triturating thick suspensions of the bacilli with sterile sand and filtering through a Berkefeld filter, the filtrate being used. Tetanus toxin was found to produce death readily. In view of the well-known extreme susceptibility of the white mouse to intraperitoneal pneumococcus inoculations, an ox-bile solution of this organism was prepared and used.

In the preparation of the pneumococcus solution a mixed culture from the three most virulent types, I, II, III, was injected into the peritoneal cavities of 6 white mice, and upon their death (approximately twenty-four hours later) saline washings of the peritoneal cavities were collected. A good thick suspension of organisms of high virulence was thus obtained. To this suspension was added several cubic centimeters of sterile ox bile. This mixture was allowed to remain in the icebox until the next day. Microscopic

examination showed the pneumococci to be practically all dissolved, those remaining were swollen and distorted

All injections were made into the peritoneal cavity with a 1 cc tuberculin syringe and short needle, the site of injection being first sterilized by saturation with iodine alcohol. Injected animals were marked with methylene blue stain.

A very satisfactory suspension of lymphocytes in vitro was obtained as follows. Mesenteric lymph nodes were removed aseptically from a rabbit. These were cut up and the pieces teased out in warm solution. After agitating the mixture until it appeared quite milky, the supernatant fluid was decanted into another warm, sterile container. Examination of this suspension showed the cellular content to be approximately 90-95 per cent lymphocytes. The balance of the cells were macrophages and erythrocytes. Fragments of lymph node stroma were present. On previous trials fairly good suspensions were obtained from the thymus. The lymphocytes could be kept alive for an indefinite period in the incubator. Amoeboid motion of the lymphocytes continued for some time as shown by warm stage examinations. Lymphocyte suspensions were also obtained from other sources and studied. These included lymph nodes from mice, rats, guinea pigs and cattle, and on one occasion, thoracic duct contents from a dog. In this instance cannulation of the thoracic duct in the cervical region was not successful but considerable fluid was obtained from the cisterna chyli. The lymphocyte content here was similar to that observed by Haynes and Field<sup>8</sup> (1931) and other workers. The former found the total white cell count per cubic millimeter of thoracic duct lymph from the dog to vary from 500 to 12,250 with about 95 per cent small lymphocytes, 4 per cent large lymphocytes and occasional polys. This method would in addition to furnishing a suspension containing lymphocytes, leave them in their natural media.

Dilutions of the toxin were made up so that the dose was contained in an amount not to exceed 0.50 cc, which pretty well fills up the peritoneal cavity of a white mouse. It was thus necessary to concentrate the lymphocyte suspension to a semisolid consistency in the higher dosages. This was accomplished by centrifuging and then decanting the supernatant fluid.

The weight of the animals injected, time of injection, identification markings, time of death, and other pertinent data were carefully recorded. It was impossible to keep the animals under constant observation. Mice were found dead in the cage at times, and note was made of the post mortem changes in order to ascertain more nearly the actual time of death. Autopsies were made on all dead animals.

*Observations and Discussion*—The first experiment comprised a series of six guinea pigs injected with diphtheria toxin. Three of these animals received the untreated toxin, and three received toxin subjected to one hour treatment with lymphocytes in vitro. The guinea pigs, receiving dosages approximating the MLD of the toxin (determined by the Parke Davis Co. on 250 gm guinea pigs) calculated on the basis of 0.000048 cc per gram body weight, all died within two or three days. The weight of these animals ranged from 704 gm to 820 gm, and yet they died in less time than that required to kill a

250 gm. guinea pig (three to four days). This is interesting as it shows that young guinea pigs tolerate diphtheria toxin better than the adults. It also shows that the lymphocyte treatment had little, if any, effect. One 627 gm. guinea pig, injected with 0.50 gm. of untreated toxin, died in one day and twenty-one hours. Another guinea pig, weighing 693 gm., received 0.066 c.c. of lymphocyte treated toxin, about one-seventh as much, and lived one day and ten hours.

Table I contains a summary of the experimental data on this series.

TABLE I  
RESULTS OF INJECTIONS OF GUINEA PIGS WITH DIPHTHERIA TOXIN

GUINEA PIG	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	TIME OF DEATH	SURVIVAL TIME HR.
Brown	704	0.033	3:00 P.M. 11/6	8:00 A.M. 11/9	65
Black	716	0.034	3:00 P.M. 11/6	1:00 P.M. 11/9	70
Spotted	627	0.500	3:00 P.M. 11/6	12:00 A.M. 11/8	45

RESULTS OF INJECTIONS OF GUINEA PIGS WITH DIPHTHERIA TOXIN—LYMPHOCYTE TREATMENT ONE HOUR

	WT.	DOSE	TIME OF INJECTION	TIME OF DEATH	SURVIVAL
Black shoulders	744	0.035	4:00 P.M. 11/22	8:00 P.M. 11/24	52
Gray	820	0.039	4:00 P.M. 11/22	8:30 P.M. 11/24	52.5
Brown	693	0.066	4:00 P.M. 11/22	2:00 A.M. 11/24	34

Autopsies of guinea pigs showed a generalized hemorrhagic appearance of the viscera, especially involving the lungs and alimentary tract. The peritoneal surface of the stomach had a purplish appearance, while the mucosa was covered with a thick, bloody exudate. The intestines, markedly distended by gas, contained also a considerable amount of frothy sanguineous material. The spleen was swollen, of a deep purple color, and the cut surface bulged. The liver showed moderate congestion. The respiratory passages and lungs contained a sanguineous exudate. All bodies were examined shortly post mortem, while still warm.

TABLE II  
RESULTS OF INJECTION WITH TETANUS TOXIN

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	CORRECT- ED SUR- VIVAL TIME
One spot	22	0.025	3:00 P.M. 4/3	8:00 A.M. 4/7	89	83
Two spots	23	0.05	3:00 P.M. 4/3	4:00 P.M. 4/5	49	49
Three spots	22	0.10	3:00 P.M. 4/3	5:30 P.M. 4/4	50.5	50.5
One streak	24	0.20	3:00 P.M. 4/3	8:00 A.M. 4/4	17	17
Two streaks	19	0.30	3:00 P.M. 4/3	8:00 A.M. 4/4	17	17
Blank	20	0.50	3:00 P.M. 4/3	9:00 P.M. 4/3	6	6

*Explanation of Corrections:* One spot mouse found dead in cage at 8:00 A.M., showed considerable post-mortem changes. Death time corrected to 2:00 A.M. When observed at 10:30 on the previous evening, this mouse showed extreme nervous symptoms with some impairment of locomotion. Two streaks mouse was found dead in cage at 8:00 A.M., and it also showed considerable post-mortem changes, so a similar correction was made in the time of death.

In the second experiment white mice were injected with tetanus toxin in dosages varying from 0.025 to 0.60 c.c. Six of them received untreated toxin and six received toxin previously subjected to lymphocytic treatment for one hour. Of the two controls used in the last group, one received lymphocyte

suspension, and the other the original untreated toxin. The first control covered possible lymphocyte protein anaphylactic reaction the second, changes occurring in the toxin after the first injection. No effect was observed from the lymphocyte protein, and there was little if any, change in the toxin. The survival time of the mice killed by the lymphocyte treated toxin showed an average increase of thirty five hours over that of the mice killed by the untreated toxin. One mouse, weighing 22 gm. was killed in eighty three hours by 0.025 cc of untreated toxin, while another 19 gm mouse receiving the

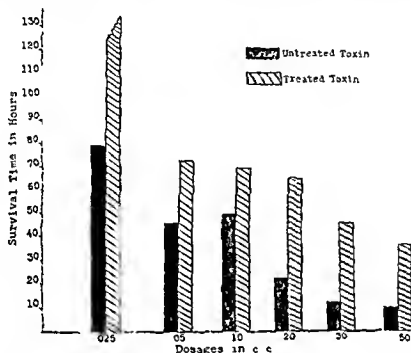


Chart 1—A comparison of the survival time of white mice injected with untreated and lymphocyte treated tetanus toxin

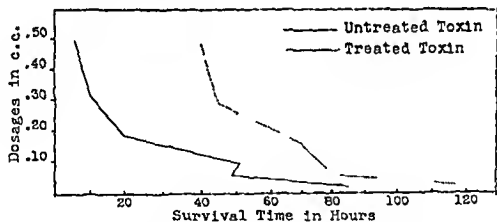


Chart 2—A comparison of the survival time of white mice injected with untreated and lymphocyte treated tetanus toxin

same dose of lymphocyte treated toxin was symptom free at one hundred twenty hours. Survival after one hundred twenty hours (five days) was taken as complete exemption from lethal effects of the toxin.

The date and results of this experiment are summarized in Tables II and III and Charts 1 and 2.

In the third experiment white mice were injected with pneumococcus toxin (ox bile solution of the organisms). They received dosages varying from 0.05 cc to 0.50 cc, five mice getting untreated toxin and five toxin subjected to lymphocyte treatment for one hour. Three controls were used, one on the first series and two in the second, they received ox bile (0.50 cc),

TABLE III

RESULTS OF INJECTION WITH TETANUS TOXIN AFTER LYMPHOCYTE TREATMENT OF ONE HOUR

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	COR- RECTED SURVIVAL TIME
One spot	19	0.025	12:30 P.M. 4/19		120	120
Two spots	22	0.05	12:30 P.M. 4/19	8:30 P.M. 4/22	80	80
Three spots	22	0.10	12:30 P.M. 4/19	4:30 P.M. 4/22	76	76
One streak	23	0.20	12:30 P.M. 4/19	8:00 P.M. 4/22	67.5	67.5
Two streaks	24	0.30	12:30 P.M. 4/19	10:00 A.M. 4/21	45.5	45.5
Speckled (Controls)	20	0.50	12:30 P.M. 4/19	8:00 A.M. 4/21	43.5	39.5
Blank	23	0.50	12:30 P.M. 4/19		120	120
Blue tail	22	0.50	12:30 P.M. 4/19	8:00 P.M. 4/19	7.5	7.5

*Explanation of Corrections:* Speckled mouse found dead in cage on April 21 at 8:00 A.M. Post-mortem changes were present, but not marked; the time of death was corrected to 4:00 A.M. Since no post-mortem changes were present when the other mice of this series were found dead, the time of death was not corrected.

*Controls:* Blank mouse received injection of lymphocyte suspension. Blue tail mouse received injection of original untreated toxin.

TABLE IV

RESULTS OF INJECTION WITH PNEUMOCOCCIC SUSPENSION IN OX-BILE SOLUTION

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	COR- RECTED SURVIVAL TIME
One spot	23	0.05	4:00 P.M. 4/10	8:00 P.M. 4/13	76	76
Two spots	20	0.10	4:00 P.M. 4/10	8:00 A.M. 4/11	16	16
Three spots	22	0.20	4:00 P.M. 4/10	8:00 A.M. 4/11	16	12
One streak	24	0.30	4:00 P.M. 4/10	8:00 P.M. 4/10	4.5	4.5
Two streaks (Control)	21	0.50	4:00 P.M. 4/10	8:00 P.M. 4/10	4.5	3.5
Blank	20	0.50	4:00 P.M. 4/10		120+	

*Explanation of Corrections:* Three spots mouse showed moderate post-mortem changes, hence time of death more nearly approximated if placed at 4:00 A.M. Two streaks mouse showed more rigor mortis than one streak mouse which was still warm; hence death time is estimated to be about one hour earlier, 7:30 P.M.

*Control:* Blank mouse received 0.50 c.c. of sterile ox-bile solution.

TABLE V

RESULTS OF INJECTION WITH PNEUMOCOCCIC SUSPENSION IN OX-BILE SOLUTION—LYMPHOCYTE TREATMENT ONE HOUR

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	COR- RECTED SURVIVAL TIME
One spot	22	0.05	12:00 A.M. 4/19		120	120
Two spots	23	0.10	12:00 A.M. 4/19		120	120
Three spots	21	0.20	12:00 A.M. 4/19		120	120
One streak	22	0.30	12:00 A.M. 4/19	10:00 A.M. 4/21	46	40
Two streaks	24	0.50	12:00 A.M. 4/19	8:00 A.M. 4/20	20	14
(Controls)						
Blank	22	0.50	12:00 A.M. 4/19		120	120
Blue tail	23	0.50	12:00 A.M. 4/19		5	5

*Explanation of Corrections:* Two streaks mouse showed considerable post-mortem changes; hence time of death was corrected to 2:00 A.M.

*Controls:* Blank mouse received 0.50 c.c. of lymphocyte suspension and blue tail mouse received 0.50 c.c. of the original untreated toxin.

lymphocyte protein (0.50 c.c.), and original untreated toxin (0.50 c.c.), respectively. No toxic effects from the bile, lymphocyte protein, anaphylactic reaction, or marked alteration in the toxin potency were observed. The untreated toxin showed high lethal potency—0.05 c.c. killing a 23 gm mouse in seventy-six hours and 0.50 c.c. killing a 21 gm mouse in three and five tenths hours.

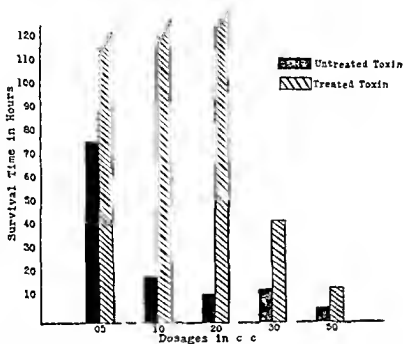


Chart 3—A comparison of the survival time of white mice injected with untreated and lymphocyte treated pneumococcus toxin (ox bile solution of organism)

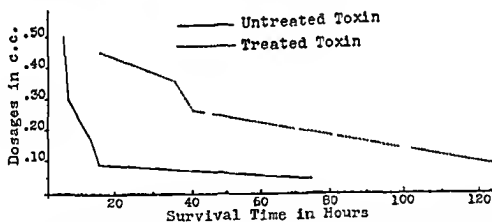


Chart 4—A comparison of the survival time of white mice injected with untreated and lymphocyte treated pneumococcus toxin (ox bile solution of organism)

There was a marked reduction of potency shown in the lymphocyte treated toxin. The mice receiving less than 0.30 c.c. survived beyond one hundred twenty hours, and the other two receiving the highest dosages, lived thirty five and five tenths and ten and five tenths hours respectively. From this it would appear that lymphocytes were much less effective in their action in the presence of high concentration of toxin.

The data and results of this experiment are summarized in Tables IV and V and Charts 3 and 4.

#### CONCLUSIONS

1 From these experimental results it would appear that lymphocytes have some deleterious effects on bacterial toxins.



2. It would not be beside the question to conclude that material contained in the lymphocyte suspension may have exerted a direct chemical protective action when absorbed by the mice (e.g., due to cholesterol content) or have acted in the nature of complement in the immunity mechanism. Further laboratory work would be necessary to decide these points.

3. It would appear that the vital action of the lymphocyte, if any, is impaired by the higher concentration of pneumococcus toxin. Tetanus toxin seems to have less effect in this respect (see Charts 1 and 3).

4. The question of the specific effect of lymphocytes on toxins is by no means decided by this preliminary work. Future work should involve repetition of the preceding experiments, as well as correction of all chances for error (e.g., pH) in the procedure. It is suggested that the effect of lymphocytes on the products of tissue necrosis, and autolysis be tested.

5. Available literature on the role of the lymphoid tissue, and particularly the lymphocyte, is suggestive, but inadequate to decide the question.

6. In the light of our present knowledge, a part in the general provision for immunity in the body is a much more likely role than a nutritive role alone, as proposed by a number of workers. Further studies should be pursued along this line.

7. In view of the latest conception of the chemical nature of certain antibodies (Marrack<sup>9</sup> and Felton<sup>10</sup>) it might be interesting to know something about the globulin content of lymphoid tissue in health and disease. The lymphoid tissue appears to be adapted to antibody production both in its anatomic distribution and its histologic structure.

8. The lymphocyte probably plays an important part in the immunity mechanism by affixing toxic cell metabolites and especially bacterial toxins, producing in turn by this reaction antitoxic substances. These substances may be liberated either as a secretory product or by autolysis of the lymphocyte. Just as the polymorphonuclear leucocyte and other phagocytic cells which engulf particulate matter in the defense reaction, the lymphocytes react apparently in defense against toxins.

#### REFERENCES

1. Virehou, Rud: Cellular Pathology (English Translation), London, 1858, p. 220.
2. Boyd, William: Pathology of Internal Diseases, Philadelphia, 1932, Lea & Febiger, pp. 646, 675.
3. Mallory, F. B.: Principles of Pathology and Histology, Philadelphia, 1914, pp. 38, 43.
4. Gulland, G. L.: The Nature, Relations, Origin and Function of Lymphocytes, *Brit. M. J.* 2: 582, 1904.
5. Pappenheimer, Alvin M.: The Reactions of Lymphocytes Under Various Experimental Conditions, *J. Exper. Med.* 25: 633, 1917; The Action of Immune Sera Upon Lymphocytes and Small Thymus Cells, *Ibid.*, p. 635.
6. Pappenheimer, Alvin M.: Experimental Studies Upon Lymphocytes, Cytotoxins for Thymus and Tonsil Lymphocytes, *Proc. N. Y. Path. Soc.* 17: 23, 1917.
7. Alexeieff, A.: Role of Lymphocytes, *Mesenchyma Theory*, *Compt. rend. Soc. de Biol.* 93: 494, 1925.
8. Metchnikoff, Elie: Immunity in Infective Diseases, London, 1907, Cambridge Univ. Press, pp. 16, 339.
9. Haynes, F. W., and Field, M. E.: Blood Cells in Dog Lymph, *Am. J. Physiol.* 97: 32, 1931.
10. Marrack, J. R.: The Chemistry of Antigens and Antibodies, Medical Research Council, Special Report Series, No. 194, London, 1934.
11. Felton, L. D.: Pneumococcus Antibodies—What Are They? *Science* 79: 277, 1934.

## THE TAKATA ARA REACTION IN AMYLOIDOSIS\*

ALFRED TARAN, B A., AND SAMUEL LIPSTIN, M D  
STATEN ISLAND, N Y

SINCE the introduction of the Takata Ara<sup>1</sup> reaction in 1925 as a laboratory test for the differentiation of lobar pneumonia and bronchopneumonia, and its utilization as a diagnostic agent for liver cirrhosis in 1929 (Stanb,<sup>2</sup> Jezler<sup>3</sup>), numerous investigations have been reported in the literature concerning the various aspects of the reaction in cirrhosis of the liver and many widely varying clinical conditions. The early reports (Skouge,<sup>4</sup> Crane,<sup>5</sup> Oliva and Pescarmona,<sup>7</sup> Ragins<sup>8</sup>) corroborated Jezler's observations of the specificity of the reaction for hepatic cirrhosis. More recent reports (Kirk,<sup>9</sup> Bowman and Bray<sup>10</sup>) show that the reaction is not specific for liver cirrhosis but that positive reactions can be obtained in a wide variety of conditions.

At the present time the rationale of the reaction is not well understood, but it is apparently related in some manner to the serum proteins. Takata and Jezler stated that it was due to the decreased stability of the serum proteins of the colloidal system which causes precipitation of the mercuric oxide and to an increase of the globulin with an inversion of the albumin globulin ratio. According to Necole,<sup>11</sup> the albumin fraction exerts a protective action, thus preventing the flocculation of the colloidal solution of the mercuric oxide. Kirk<sup>9</sup> investigated this relationship in a series of 56 patients. In 18 cases with a reversed albumin globulin ratio, 13 gave positive Takata Ara reactions. Tabulating the percentage increase of the globulin fraction, he found 21 such cases, and the Takata Ara reaction was positive in 19 of them. It was his opinion that the reversal of the albumin globulin ratio was not the important factor and that the Takata Ara reaction was likely to be positive in any disease in which the globulin level was elevated. Ragins<sup>8</sup> was unable to find any correlation between the test and the amount of albumin, globulin, or the albumin globulin ratio. Bowman and Bray,<sup>10</sup> on the other hand, reported that positive reactions are likely to occur in all conditions in which the total protein content of the blood serum and the albumin globulin ratio were low.

Since the Takata Ara reaction has been used so extensively in liver pathology and has some apparent relationship to the serum protein fractions, the test has been used frequently at this hospital on patients suffering from a generalized amyloidosis complicating pulmonary tuberculosis. In the large majority of cases flocculation of the mercuric oxide occurred and the tests were reported as strongly positive. Israel and Reinhold<sup>12</sup> have recently reported three cases of amyloidosis in which the Takata Ara test had been performed. One of these cases showed a negative reaction, one was slightly positive, and the third strongly

\*From the Department of Pathology, Sea View Hospital, Staten Island. Dr. Oscar Auerbach, Director.

Received for publication June 16, 1938.

positive. In the third case blood protein determinations were made and revealed an inversion of the albumin and globulin fractions, with an albumin globulin ratio of less than 1.3. Because of the usual extensive involvement of the liver in generalized amyloidosis and the alterations of the protein fractions of the blood serum in the later stages of amyloidosis with the development of amyloid nephrosis, albuminuria, and edema, it was decided to correlate this reaction along with the total serum proteins, albumin, globulin, and albumin-globulin ratios in a large group of patients with amyloidosis and a control group without amyloidosis.

This study consisted of 40 cases of amyloidosis and 25 control cases without amyloidosis. All the patients were adults with advanced pulmonary or extrapulmonary tuberculous and all cases were excluded in which there was evidence of other metabolic disorders, such as diabetes mellitus, the nephritides, or tuberculous involvement of the kidneys, with the exception, of course, of amyloidosis of the kidney in the amyloid group. In every case a Congo red test, a Takata-Ara test, and blood protein determinations were performed. The main criterion for the diagnosis of amyloidosis in the one group was a 100 per cent absorption of Congo red. Through a recent evaluation of the Congo red test in amyloidosis by one of us (S. L.<sup>12</sup>), it was determined that extensive amyloid involvement (especially of the liver) was a necessary condition for the complete absorption of the dye in one hour. Other clinical manifestations of amyloidosis were also present in most instances, such as an albuminuria ranging from a trace to four plus, and enlarged firm palpable livers and spleens. However, it is well known that extensive amyloidosis of both the liver and the spleen can be present in organs of normal or even subnormal size which are not palpable, and that the albuminuria is in direct relationship to the degree of kidney amyloid involvement. In the control group the percentage of Congo red dye absorption was well within the normal limits and no clinical evidence of amyloidosis was present.

#### METHODS

The Takata-Ara test employed in this study was essentially the one described by Heath and King,<sup>14</sup> with the exception of slight changes in the reading of the results. One cubic centimeter of 0.9 per cent sodium chloride solution is added to each of six test tubes (100 by 13 mm.). To the first tube, 1 c.c. of serum to be tested is then added and the contents are thoroughly mixed. One cubic centimeter of this mixture is removed and added to the next tube, and the contents thoroughly mixed. The process is repeated in the remaining tubes, the 1 c.c. from the sixth tube being discarded. The dilutions of serum are 1:2, 1:4, 1:8, 1:16, 1:32, 1:64. To each tube is added 0.25 c.c. of 10 per cent sodium carbonate solution, and 0.15 c.c. of 0.5 per cent mercuric chloride solution. The mixtures are allowed to stand at room temperature and readings are made from sixteen to twenty-four hours later.

The criteria for positive and negative reactions as used by Heath and King are the following: reactions showing complete precipitation in one tube or some flocculation in five tubes were considered strongly positive; those showing

almost complete precipitation in one tube or flocculation in three tubes were considered positive, a weakly positive reaction was indicated when there was a slight but definite flocculation in one or two tubes. Doubtful flocculations in any two tubes were recorded as suspicious. Negative reactions were those in which no flocculent precipitation was evident.

TABLE I  
DATA OF THE AMYLOID GROUP OF CASES

CASE	TAKATA ARA REACTION	TOTAL PROTEINS	ALBUMIN GM PER CENT	GLOBULIN GM PER CENT	A/G RATIO
1	4+	64	37	27	1.37
2	3+	56	29	27	1.07
3	1+	62	36	26	1.38
4	3+	56	28	28	1.00
5	Negative	58	37	21	1.76
6	1+	56	29	27	1.07
7	1+	59	31	28	1.10
8	2+	60	32	28	1.14
9	Negative	60	35	25	1.40
10	1+	58	34	24	1.41
11	4+	57	29	28	1.03
12	Negative	60	36	24	1.50
13	2+	58	34	24	1.41
14	2+	58	32	26	1.23
15	1+	58	30	28	1.07
16	2+	57	30	27	1.11
17	Negative	61	37	24	1.54
18	Negative	68	45	23	1.95
19	4+	70	40	30	1.33
20	4+	63	37	26	1.42
21	1+	68	40	28	1.42
22	Negative	60	34	26	1.30
23	4+	56	29	27	1.07
24	Negative	61	39	22	1.77
25	2+	55	26	27	1.03
26	4+	65	32	33	0.96
27	4+	56	28	28	1.00
28	4+	57	29	28	1.03
29	3+	51	22	29	0.75
30	1+	55	29	26	1.11
31	3+	53	26	27	0.96
32	2+	59	33	26	1.26
33	2+	64	35	29	1.20
34	4+	62	29	33	0.87
35	1+	68	42	26	1.61
36	2+	62	33	29	1.13
37	Negative	67	45	22	2.04
38	1+	61	35	26	1.34
39	1+	57	29	28	1.03
40	Negative	67	44	23	1.91

In our present study we have found it to be more practical to record the positive reactions as four plus, three plus, two plus, and one plus, in order to further subdivide the strongly positive reactions and to facilitate the reading of the tables. We have regarded the strongly positive reaction described by Heath and King as a three plus reaction, the four plus being denoted by complete precipitation in more than one tube and flocculation in the remaining tubes. Whereas they have recorded doubtful reactions as suspicious, we have considered them to be negative.

Taran's<sup>15</sup> modification of the Bennhold method was employed in the Congo red test. This consisted of an acetone extraction of the dye prior to the colorimetric reading. Blood proteins were determined by Howe's<sup>16</sup> micro-Kjeldahl method. The normal range for the serum proteins is considered to be the following: total serum proteins, 6.0 to 8.5 per cent; albumin, 4.5 to 6.0 per cent; globulin, 1.5 to 2.5 per cent. The normal albumin-globulin ratios generally range from 1.8 to 2.5.

## RESULTS

Positive Takata-Ara reactions were present in 31 of the 40 cases in the amyloid group, an incidence of 77.5 per cent (Table I). These cases showed in general, a low albumin fraction with a corresponding increased globulin fraction. The albumin-globulin ratios ranged from 0.75 to 1.61. Negative Takata-Ara reactions were recorded in the remaining 9 cases of this group. In these cases the albumin-globulin ratios ranged from 1.30 to 2.04.

In the nonamyloid control group of 25 cases, positive Takata-Ara reactions were recorded in only five instances, an incidence of 20 per cent (Table II).

TABLE II  
DATA OF THE NONAMYLOID GROUP OF CASES

CASE	TAKATA-ARA REACTION	TOTAL PROTEINS	ALBUMIN GM. PER CENT	GLOBULIN GM. PER CENT	A/G RATIO
1	Negative	6.8	4.5	2.3	1.95
2	Negative	6.0	3.9	2.1	1.85
3	Negative	6.5	4.2	2.3	1.82
4	Negative	6.7	4.5	2.2	2.04
5	Negative	6.9	4.1	2.8	1.46
6	Negative	8.3	6.0	2.3	2.60
7	1+	6.2	3.5	2.7	1.29
8	Negative	6.2	3.9	2.3	1.69
9	1+	6.5	3.3	3.2	1.03
10	Negative	7.4	4.8	2.6	1.84
11	Negative	7.2	4.7	2.5	1.88
12	3+	10.6	3.6	7.0	0.51
13	Negative	6.8	4.3	2.5	1.72
14	2+	7.7	4.1	3.6	1.13
15	Negative	7.2	4.9	2.3	2.13
16	Negative	7.3	5.2	2.1	2.47
17	Negative	7.0	4.9	2.1	2.33
18	Negative	7.5	5.2	2.3	2.26
19	Negative	7.2	5.0	2.2	2.27
20	Negative	7.4	5.3	2.1	2.52
21	Negative	7.1	5.1	2.0	2.55
22	Negative	7.0	4.9	2.1	2.33
23	Negative	6.8	4.6	2.2	2.69
24	1+	6.8	4.2	2.6	1.61
25	Negative	7.1	4.9	2.2	2.22

In these cases the albumin-globulin ratios were low, ranging from 0.51 to 1.61. In the 20 cases in which the Takata-Ara reactions were negative, the blood proteins were generally within normal limits and the albumin-globulin ratios ranged from 1.46 to 2.60.

Since the inception of this study, 10 cases have come to autopsy. In 4 of these cases the liver, kidney, and adrenal glands were

Cases 4, 7, 8, in the amyloid group have extensive amyloidosis of the liver, kidney, and adrenal glands present.

## DISCUSSION

In this study one is impressed with the high incidence (77.5 per cent) of positive Takata Ara reactions in the amyloid group as compared with the relatively low percentage (20 per cent) in the control group in which no amyloidosis was present. The question immediately arises as to whether these results can be attributed to the physiologic and pathologic changes (especially in the liver) as the result of the replacement of the parenchyma by amyloid, or whether they are the result of quantitative alterations in the serum proteins secondary to the amyloidosis. If the positive Takata Ara reactions are the direct result

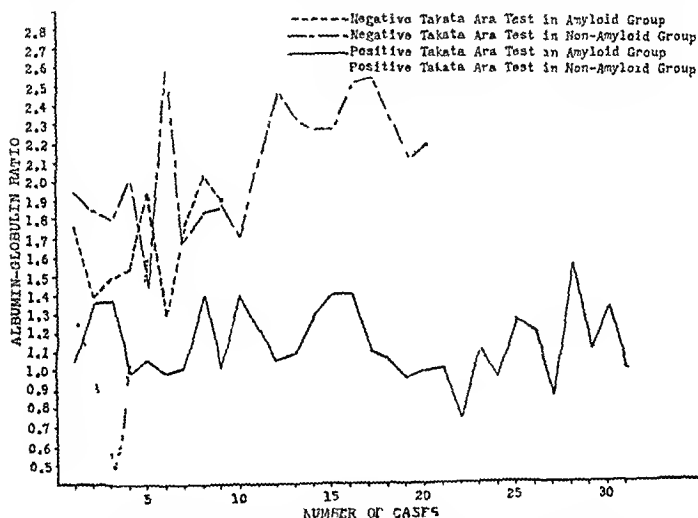


Fig 1—Correlation between the Takata Ara reaction and the albumin globulin ratio

of the amyloid changes how can we account for the positive reactions in those cases in which no amyloidosis was present or the negative reactions in the presence of amyloidosis? There is apparently no relationship between the amyloidosis per se and the Takata Ara reaction.

On the other hand, there appears to be a close relationship between the serum proteins, especially the albumin globulin ratio, and this reaction. The Takata Ara reaction was positive in practically all instances in which the albumin globulin ratio was below 1.5, both in the amyloid and nonamyloid groups. This relationship is demonstrated in Fig 1. In all instances, with two exceptions in which the albumin globulin ratio was below 1.5, the Takata Ara reaction was positive, and in all instances with three exceptions, in which the albumin globulin ratio was above 1.5, the reaction was negative. These findings are in close accord with those of Israel and Reinhold,<sup>17</sup> who found the incidence of abnormal low albumin globulin ratios almost identical with that of positive Takata Ara reactions.

## SUMMARY AND CONCLUSIONS

1. The relationship between the Takata-Ara reaction and amyloidosis was studied in a group of 40 tuberculous patients. In each instance the Takata-Ara reaction, total proteins, albumin, globulin, and albumin-globulin ratios were determined. Similar examinations were also performed on a control group of 25 tuberculous patients with no evidence of amyloidosis.

2. Positive Takata-Ara reactions were recorded in 31 of 40 cases (77.5 per cent) of the amyloid group as compared with only 5 of the 25 control cases (20 per cent).

3. The albumin levels were generally lower than normal, with corresponding increase of the globulin fractions in those cases with positive Takata-Ara reactions.

4. The Takata-Ara reactions were positive in all but two instances in which the albumin-globulin ratios were lower than 1.5, and negative in all but three instances in which the albumin-globulin ratios were above 1.5, both in the amyloid and nonamyloid groups.

5. There is apparently no direct relationship between the presence of amyloidosis per se and a positive Takata-Ara reaction. The positive reaction from this study rather appears to be dependent upon the alterations of the albumin and globulin fractions and is in close correlation with the albumin-globulin ratio.

## REFERENCES

1. Takata, M.: Über eine Kolloidchemische Sero-Diagnostik der Lungenentzündung, Tr. Sixth Congress Far Eastern Assn. Trop. Med. 1: 693, 1925.
2. Takata, M., and Ara, K.: Über eine Neue Kolloidchemische Liquorreaction und Ihre Praktischen Ergebnisse, Ibid. 1: 667, 1925.
3. Staub, H.: Über Functionelle Leberdiagnostik, Schweiz. Med. Wehnschr. 10: 305, 1929.
4. Jezler, A.: Die takatasehe Kolloidreaktion in serum und Körperflüssigkeiten und ihre Beziehung zu storungen den Eiweisstoffwechsels der Leber, Ztschr. f. klin. Med. 114: 739, 1930.
5. Skouge, E.: Der Wert der Takata-Ara Reaktion und der Koagulationsbandbestimmung bei Leberkrankheiten, Klin. Wehnschr. 12: 905, 1933.
6. Crane, M. P.: A Modified Mercuric Chloride Reaction (Takata-Ara) in Cirrhosis and in Neoplasms of the Liver, Am. J. M. Sc. 187: 705, 1934.
7. Oliva, G., and Pescarmona, M.: The Takata-Ara Reaction in Hepatic Diseases, Minerva med. 2: 12, 1933.
8. Ragins, A. B.: The Value of the Takata and Ara Reaction as a Diagnostic and Prognostic Aid in Cirrhosis of the Liver, J. LAB. & CLIN. MED. 20: 902, 1933.
9. Kirk, R. C.: The Takata-Ara Test and Its Relation to Cirrhosis of the Liver, J. A. M. A. 107: 1354, 1936.
10. Bowman, R. O., and Bray, R. S.: An Evaluation of the Takata-Ara Reaction for Diagnosis of Liver Cirrhosis, J. LAB. & CLIN. MED. 22: 532, 1937.
11. Neele, R.: Ztschr. f. klin. Med. 110: 94, 1929.
12. Israel, H. L., and Reinhold, J. G.: Detection of Cirrhosis and Other Diseases of the Liver by Laboratory Tests, J. LAB. & CLIN. MED. 23: 588, 1938.
13. Lipstein, S.: An Evaluation of the Congo-Red Test for Amyloidosis, Am. J. M. Sc. 195: 205, 1938.
14. Heath, C. W., and King, E. F.: The Takata-Ara Test in the Diagnosis of Liver Disease, New England J. Med. 211: 1077, 1934.
15. Taran, A.: A Further Improved Congo Red Test for Amyloidosis, J. LAB. & CLIN. MED. 22: 975, 1937.
16. Howe, P. E.: The Determination of Proteins in Blood—A Micro Method, J. Biol. Chem. 49: 109, 1921.

# SOME EFFECTS PRODUCED IN THE NORMAL STOMACH BY THE INGESTION OF MODERATE AND MASSIVE QUANTITIES OF ALUMINUM HYDROXIDE GEL\*

J P QUIGLEY, PH D, I H EINSEL MD AND I MESCHAN, MS  
CLEVELAND, OHIO

CLINICAL improvement of peptic ulcer has been reported to follow the ingestion of aluminum hydroxide<sup>1-11</sup> The mechanism by which the therapeutic effects are elicited has not been established although the following possibilities deserve serious consideration neutralization of gastric acid, exertion of an astringent action on the mucosa especially in the vicinity of the ulcer, formation of a protective coating over the ulcerated tissue, augmentation of mucus production, modifications in gastric motility or in gastric evacuation, depression of gastric secretory activity by reducing the secretory capacity or decreasing the stimulating effect of food in the gut (interfere with production of secretagogues or gastrin) The present investigation was undertaken to study certain of these possibilities and to ascertain to what extent they might explain the therapeutic value of the preparation Undesirable effects from the therapeutic use of aluminum hydroxide have generally been denied but the possibility of such action must be admitted especially when high dosage is employed, or special conditions permit excessive absorption of the preparation, or the subject is especially susceptible to its action Many of the desirable and undesirable effects of aluminum hydroxide administration should be demonstrable in exaggerated form by its prolonged administration in massive dosages

In the first phase of our investigation we studied chiefly the *prolonged* or more permanent effects which might persist beyond the immediate interval when the stomach contained aluminum The latter portion of our study dealt with the *immediate* effects on motility and tone of the gastric body and antrum and the pyloric sphincter and duodenal bulb which followed the introduction of aluminum hydroxide into the stomach or duodenum

## EFFECT OF LARGE DOSES ON GASTRIC EVACUATION TIME GASTRIC SECRETION, AND HISTOLOGY OF THE GASTRIC MUCOSA

*Methods*—For this investigation we employed six healthy vigorous mature dogs each weighing approximately 15 kg The animals were thoroughly trained to cooperate in the experiments, and emotional reactions were largely

\*From the Department of Physiology and Medicine at City Hospital Western Reserve University Cleveland

First reported before the Experimental Medicine Section Cleveland Academy of Medicine May 12 1933

Received for publication June 17 1938



avoided. They were submitted to laparotomy, and biopsy specimens were taken from the anterior gastric wall in the fundic body and antral regions. The specimens were immediately fixed in 10 per cent formaldehyde and stained with eosin and hematoxylin. Following complete recovery from this surgical procedure, gastric evacuation time was determined in each animal by the following method: 30 gm. of barium sulfate ("for clinical use") were thoroughly incorporated with 100 gm. of cooked corn-meal mush, passed through a sieve, and mixed with sufficient water to make 250 c.c. This preparation was administered by stomach tube twenty-four hours postcibum. Fluoroscopic determinations of gastric emptying-time were made repeatedly until results in good agreement were obtained for each animal.

In studying gastric secretion the emphasis was placed on free acid production, for acid is of paramount importance in the etiology and chronicity of peptic ulcer (Mann,<sup>12</sup> Dragstedt,<sup>13</sup> etc.). Histamine was employed as the stimulus to the gastric glands, since by this test an indication is obtained of the maximal amount of acid which the stomach can secrete (Wilhelmj<sup>14</sup>).

Each dog was studied while lying on the left side at ease on a mattress, twenty-four hours postcibum. A rubber tube of 5 mm. bore and tipped with a brass olive was introduced into the stomach via the esophagus so the olive was located in the portion of the stomach from which gastric juice flowed most freely. The animal was prevented from making chewing movements. Flow of gastric juice via the rubber tube resulted from normal intragastric pressure and siphonage. Additional juice was not obtained by the application of suction. Collection of juice by this method was satisfactory for obtaining samples characteristic of the gastric contents. It is impossible to state that all the juice secreted during the interval was collected, but the method apparently did provide a close approximation of that condition.

The juice was allowed to flow spontaneously for fifteen minutes, then 0.5 mg. of histamine was administered subcutaneously, and the secretion collected in fifteen-minute portions for a total of one hundred twenty minutes. The volume, free acid, combined acid, bile, and mucus were immediately estimated in each sample. Gastric secretion was determined by this method in each animal until results showing good agreement were obtained.

The six dogs were divided into two groups—the experimental group of three animals to receive aluminum hydroxide and the remaining animals to be retained as controls. An experimental animal and a control animal were caged together throughout the investigation and all were fed the same stock diet. The experimental dogs received gelatinous, colloidal aluminum hydroxide, prepared as follows: Aluminum chloride and sodium carbonate solutions were mixed together with stirring until the mixture was neutral to phenolphthalein and neutral red. The white, creamy gel resulting had an astringent but not an unpleasant taste. It was washed until practically free from chlorides and was considered suitable for this study only if it had a combining power when titrated with Töpfer's reagent indicated by the neutralization of 20 to 25 c.c. N/10 hydrochloric acid per c.c. of gel. Sixty cubic centimeters of this preparation were administered with 200 c.c. of water by stomach tube daily at

8 30 A M and 4 30 P M for seventy-nine consecutive days. Comparing this dose with that employed clinically (24 to 48 cc) and making allowance for the weight difference between dog and man, the dose employed was 175 to 35 times greater than the therapeutic dosage. At the termination of the medication period, gastric evacuation time and the gastric secretory response to histamine were immediately redetermined on all six animals by a second series of trials. Biopsy specimens from all animals were obtained from areas adjacent to the site of the first specimens. The gastric secretory response to histamine was again determined one month following termination of the aluminum hydroxide administration period.

*Results and Discussions*—The dogs receiving aluminum hydroxide lost approximately 500 gm in weight during the period of administration as judged by their weight preceding and following this interval by the weight changes of the control animals, and also by the rapid weight increase which followed the administration period. Administration of the preparation was considered with disfavor by the experimental animals; vomiting occurred on a few occasions, but nausea was almost invariably experienced. The animals usually salivated before (development of a conditioned reflex) and after the introduction of the stomach tube. The nausea was a transient effect of the large dosage of preparation employed and perhaps could have been avoided if divided doses had been used. It induced anorexia and thus a decreased food intake. Beazell and others<sup>15</sup> have shown that therapeutic doses of aluminum hydroxide do not decrease the digestion and absorption of food. The anorexia probably fully explains the weight loss and we have no reason to anticipate that such an effect would have followed moderate dosages. Other ill effects from the experimental procedures were not observed. The animals remained vigorous, had good coats of hair, etc. The nausea promptly disappeared, appetite returned, and a gain in weight occurred when medication was discontinued.

*Gastric Emptying Time*—Preceding the administration of aluminum hydroxide, the average of six determinations of the emptying time for each of our six dogs was (a) 3 hours, 20 minutes, (b) 3 hours, 50 minutes, (c) 4 hours, 15 minutes, (d) 4 hours, 5 minutes, (e) 3 hours, 55 minutes, (f) 4 hours, 30 minutes. Following the period of aluminum administration the evacuation time had not changed significantly for the experimental animals (a) 3 hours, 10 minutes, (b) 4 hours, 10 minutes, (c) 4 hours, 5 minutes, or for the control animals (d) 4 hours, 30 minutes, (e) 3 hours, 35 minutes, (f) 4 hours, 45 minutes. These results do not suggest any enduring effect on gastric evacuation time from prolonged administration of aluminum hydroxide in massive doses.

In a patient having hyperacidity, heart burn, and postprandial discomfort, Crohn observed a reduction in gastric emptying time and a lowering of gastric acidity when aluminum hydroxide (Alucox) was administered. Results discordant from ours are to be anticipated in his study, for in Crohn's case evacuation time and acidity were determined while the aluminum was in the stomach, and in addition the investigation was made on a stomach manifesting distinct abnormalities, i.e., in the pathologic state.

*Gastric Secretion.*—After the animals had become accustomed to the stomach tube, six determinations of gastric secretory response to histamine were made preceding and six following the aluminum administration period on each dog of both the control and experimental groups. After averaging these results, such close agreement was noted from the experimental animals during pre- and post-administration periods and also from control animals as to justify the following conclusions: The "resting" volume of gastric juice before histamine and the total juice collected during a period of one hundred and five minutes after histamine remained constant (generally within 5 per cent). The response to histamine began about fourteen minutes after its subcutaneous injection, and the maximum volume and usually the highest concentration of free acid was reached during the thirty- to forty-five-minute period following its administration. Combined acid showed a slight but definite elevation, while free acid was lowered slightly following the period of aluminum administration. Both sets of values had returned to normal when a third series of six determinations were made one month following termination of the administration period (Fig. 1). No significant modification in the quantity of bile in the gastric contents was noted, but mucus was slightly more abundant after aluminum administration. These results indicate that prolonged administration of massive doses of aluminum hydroxide or the taking of biopsy specimens did not significantly alter the gastric emptying process or the response of the gastric glands to histamine. A slight increase in the neutralization of hydrochloric acid occurred. This may have resulted from the moderately increased formation of mucus which was simultaneously noted.

It might be anticipated that prolonged administration of aluminum hydroxide would decrease the secretory activity of the gastric glands, manifesting such a change by a decrease in volume of secretion or a decrease in free acid. Such action might result from the astringent or local toxic action of aluminum hydroxide (this preparation is used as a colloidal protein precipitant) or from a systemic toxic action to the gastric mucosa following absorption of aluminum. The fact that in our experiments the secretory response to histamine was not altered in volume and only slightly reduced in free acidity, even after prolonged administration of massive doses of aluminum hydroxide, shows that these ill effects do not develop significantly.

After a period of therapy consisting of aluminum hydroxide four to six times a day, Adams, Einsel, and Myers<sup>5</sup> noted a decrease in the concentration of free acid and in the volume of gastric juice collected both in the "resting" state and after an alcohol test meal in ulcer patients. The decrease was especially marked in cases of hyperacidity, and, according to these authors, explains, at least partially, the efficacy of colloidal aluminum hydroxide. These observations do not necessarily indicate a decrease in the secretory activity of the gastric glands, but may result from the improved gastric evacuation and more effective neutralization which has frequently been reported during the remission of a peptic ulcer. The question should be investigated in Pavlov pouch animals or some similar preparation. It is also possible that the secretory re-

sponse is depressed only to the weaker types of stimuli, for Emsel, Adams, and Myers<sup>4</sup> noted reduced secretion to an alcohol test meal after aluminum therapy, but a normal response to histamine

Ivy, Terry, Pauley and Bridley<sup>16</sup> found that 20 cc. Ciemalin (aluminum hydroxide gel) or 10 gm. alucol (aluminum hydroxide solid) administered to dogs four times daily for four months produced no significant decrease in gastric secretory response to a test meal, but on the contrary, a tendency to a slight increase obtained. The lack of agreement with our findings may be explained by the employment of larger quantities of aluminum in our experiments the use

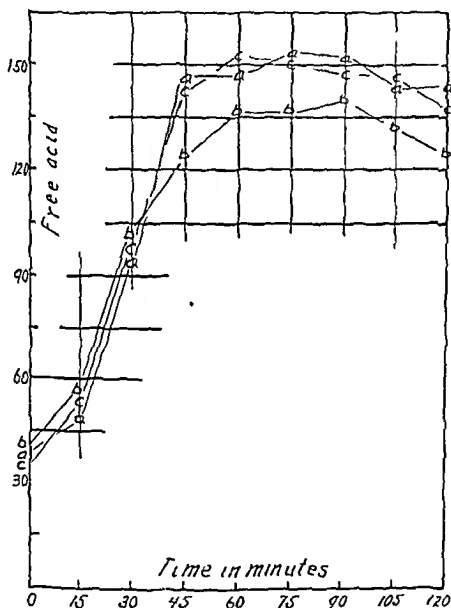


Fig. 1—Production of free acid following subcutaneous histamine at zero time. Each line is an average of six dogs. (a) immediately following seventy-nine day administration and (c) one month following termination of administration.

\* 15 mg. histamine  
aluminum  
cc. daily

of a different stimulus to the gastric glands or to the development of anorexia, etc., in our animals. The slight augmentation in gastric secretion obtained in normal dogs by Ivy and co-workers and the slight decrease in free acid we observed in normal animals after massive doses of aluminum hydroxide, show that the effect of this preparation on gastric secretion of the normal stomach is practically negligible. If the reduction in secretion is greater in ulcer cases as reported by Adams and associates, the effects evidently develop indirectly through relief of the ulcer.

*Biopsy Specimens and Considerations of Toxicity.*—Histologic examination of the portions of the anterior gastric wall showed no significant differences between those taken before and those taken after the prolonged administration of large doses of aluminum hydroxide. We are indebted to Dr. H. S. Reichle and Dr. David P. Seecof, Pathologists at City Hospital, Cleveland, for an examination of these tissues. Edema of the submucosa of unknown origin was



Fig. 2.—Photomicrographs of gastric histologic specimens taken at termination of a seventy-nine-day period of aluminum hydroxide gel administration. A, Cardiac region; B, midportion; C, pyloric antrum region. Description in text.

found in most specimens. A report on the three sections shown in Fig 2, taken during the post administration period, follows:

*No 27 Cardiac Region* The surface epithelium shows no significant change. The edematous submucosa separates the necks of the glands as previously noted. The glands are present in normal number and show no change. The capillaries are thin walled, there is no perivascular infiltration, and the musculature shows no change.

*No 27 Midportion* The edema previously noted is not present. Surface epithelium normal, glands are abundant and show no change. Musculature is of normal character. Larger vessels below the muscularis mucosae are thin walled but show no other noteworthy change.

*No 27 Pylorus (Antrum Region)* The surface epithelium descends rather deeply into the necks of the glands. There is some edema between the necks of the glands. The glandular structure is present in the normal quantity. Capillaries are thin walled and the musculature shows no significant changes.

Ivy and co workers considered the possibility of aluminum preparations producing gastritis and thus modifying gastric secretory activity. Our results indicate that massive doses of aluminum hydroxide gel given during a prolonged interval do not produce gastritis.

The absence of detectable histologic changes in our biopsy specimens, especially in the mucosa, and the absence of other injurious effects from the aluminum (we do not ascribe the nausea and weight loss during the medication period to a specific or systemic action of aluminum hydroxide) emphasize the nontoxic nature of such medication. From the review of the literature and from their own observations, Ivy and associates were also of the opinion that toxic effects are not to be anticipated from prolonged administration of aluminum hydroxide even in large quantities.

#### ACTION OF MODERATE DOSES ON GASTRIC AND PYLORIC MOTILITY

*Methods and Results*—In studying the effects of aluminum hydroxide on the gastric body and pyloric antrum region the emphasis was placed on the interval immediately after administration and subsequently for approximately an hour or so, while the aluminum was in the stomach and upper intestine.

Six normal dogs were trained to lie at ease while fasting on comfortable mattresses, and gastric motility was recorded from the stomach by a balloon introduced via the esophagus. Records were made for two hour periods, and on twelve occasions the effect was studied after injecting 50 to 100 cc 0.9 per cent sodium chloride into the stomach via one half of the two lumen tube to which the balloon was attached. The injected material was warmed at 39° C, and was administered during an interval of two minutes' duration. Saline injected into the stomach usually had no effect on gastric motility, but on two occasions contractions and tone were promptly depressed for two to ten minutes, subsequently the "hunger" motility was normal or slightly exaggerated for one hour. After making control records in twelve additional experiments, 10 cc of aluminum hydroxide gel suspended in 50 cc saline were injected into the stomach during an interval of vigorous motility under conditions similar to those previously described for the administration of saline. Usually motility

and tone were promptly inhibited for two to twelve minutes, then complete recovery obtained. The subsequent motility tended to be slightly more irregular than normal, and indications of hypermotility periods alternated with hypomotility during the one-hour period of observation. The motility changes were not of sufficient duration or magnitude to possess clinical significance.

In ten experiments 4 gm. of powdered aluminum hydroxide (Merek) suspended in 100 c.c. of water were used as the test solution. The results were, in general, similar to those from aluminum hydroxide gel. This preparation of aluminum is the one employed in ulcer cases by Einsel and Rowland<sup>3</sup> and abandoned by them because it did not neutralize hydrochloric acid and because they obtained better results from gel.

### BULB

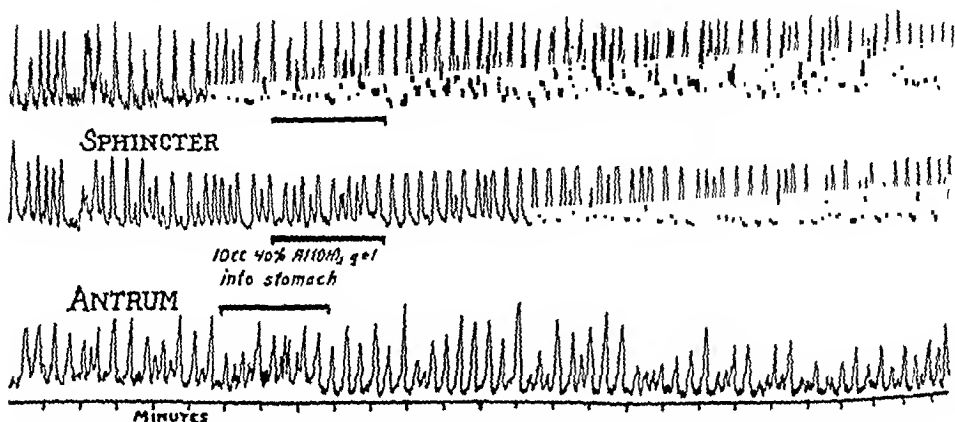


Fig. 3.—Effect on the pyloric sphincter region from administration of aluminum hydroxide gel into the stomach.

Four normal dogs were provided with permanent metal cannulae into the stomach and duodenum. Balloon records were obtained from the pyloric antrum, pyloric sphincter, and duodenal bulb of these animals according to the method described by Meschan and Quigley.<sup>17</sup> In sixteen experiments 2 to 10 c.c. quantities of aluminum hydroxide gel diluted to 40 per cent were injected on seven occasions into the stomachs and in seven trials into the duodenums of animals fasting for twenty-four hours. Full strength aluminum hydroxide gel was introduced in 10 c.c. quantities once each into the stomach and duodenum.

In six of the eight experiments involving injection of aluminum hydroxide gel into the duodenum, and in two of the eight experiments in which an intragastric injection was made, no change in motility or tone of the antrum, sphincter, or bulb resulted. In the remaining experiments very slight inhibition, sometimes involving the entire sphincter region, but on other occasions involving only a portion of this region developed in five experiments, but slight augmentation resulted in three experiments. Aluminum gel administration failed to produce any other significant deviation from the normal motility or tone of the sphincter region (Fig. 3). Even when 30 c.c. of 40 per cent gel was given in divided doses into the stomach or duodenum during a period of ninety minutes, no significant changes in tone or motility of this portion of the gut could be demonstrated.

Since changes in tone and motility of the sphincter region were meconstant in occurrence, of slight magnitude and of short duration, they appear to be insignificant. According to these results, aluminum hydroxide does not exert its therapeutic action by altering the tone or motility of the stomach, sphincter, or duodenal bulb. As interpreted by Meschan and Quigley<sup>1</sup> this should preclude a significant alteration in gastric evacuation or the development of reversed peristalsis or duodenal regurgitation during the one hour period of observation subsequent to the injection. The absence of a significant alteration in gastric evacuation here indicated from the acute action of aluminum hydroxide supplements the conclusion reached in an earlier portion of this investigation where the prolonged administration of this drug also failed to alter emptying time. However, as has been emphasized earlier in this report an absence of effect on the normal gut does not preclude a significant alteration of motility or emptying time from aluminum hydroxide in the presence of an ulcer or other pathologic condition.

#### COMMENT

In the first portion of our investigation an attempt was made to demonstrate chronic effects from aluminum hydroxide administration. No persistent modifications were found to occur in a significant degree even after the prolonged administration of this drug in massive quantities. Toxic effects did not develop, other than an anorexia and slight weight loss (apparently related to the method of administration), the subjects remained in good physical condition, histologic changes were not demonstrable in the gastric wall, and the gastric glands could respond in almost normal manner to a strong stimulus. This failure to demonstrate persistent effects may indicate that aluminum hydroxide action is largely restricted to the period when it is present in the stomach. Thus a scientific basis would be afforded for employment of the continuous aluminum hydroxide drip method of Woldman and Rowland<sup>11</sup> to provide maximum efficacy. Modifications of gastric motor and secretory activity from aluminum therapy probably cannot be anticipated beyond the immediate period of administration, except those modifications which develop indirectly through the remission of the ulcer or other pathologic state.

Our results indicate that the normal stomach is not entirely satisfactory as a test object to investigate the therapeutic mechanism of aluminum hydroxide, since apparently this drug produces some effects in the ulcer bearing subject not obtained in the normal. We failed to demonstrate a significant change in motility of the stomach or pyloric sphincter region in gastric evacuation time or the volume of gastric juice either "resting" or subsequent to histamine administration. The secretion of free acid was reduced and of combined acid was increased in response to histamine, but not significantly. Clinical reports, on the contrary, indicate a shortening of evacuation time<sup>1</sup> and a marked reduction in gastric secretion, both volume and concentration of free acid.<sup>5</sup> The efficacy of aluminum hydroxide in clinical conditions may therefore, depend on an action limited to the pathologic gut and not demonstrable on normal tissue. The therapeutic dose of aluminum hydroxide gel is 4 c.c. six times daily. This quantity would completely neutralize 600 c.c. of gastric juice containing 0.4 per cent hydrochloric acid (about the volume secreted in response to a meal).



It would also reduce the acid content of 1,500 c.c. of such juice to 0.15 per cent hydrochloric acid, which Dragstedt<sup>13</sup> has emphasized is the critical level below which gastric juice has no digestive or corrosive action. The significant decrease in gastric acidity produced by this regimen may largely explain the therapeutic value of aluminum hydroxide.

#### SUMMARY

The administration of aluminum hydroxide gel in massive doses (120 c.c. daily) for seventy-nine days to normal dogs was without significant effect on gastric evacuation time, or the histologic structure of gastric tissue, and only transiently reduced the gastric secretory response of free acid to histamine.

Quantities of aluminum hydroxide within the clinical range failed to produce significant changes in motility or tone of the stomach or pyloric antrum region as indicated by balloon records made while the medication was introduced into the stomach or duodenum.

Reasons are given for believing the therapeutic action of aluminum hydroxide depends largely on its buffering action toward gastric acid and on significant modifications in gastric evacuation and secretory activity demonstrable in the presence of ulcer or other pathology, but not in the normal gut.

#### REFERENCES

1. Crohn, B. B.: The Clinical Use of Colloidal Aluminum Hydroxide as a Gastric Antacid, *J. LAB. & CLIN. MED.* 14: 610, 1929.
2. Kreis, L.: Kausale Radikalheilung der Hyperazidität der konservative Behandlung. *Wien. med. Wchnschr.* 81: 140, 1931.
3. Einsel, I. H., and Rowland, V. C.: Aluminum Hydroxide Treatment of Peptic Ulcer, *Ohio State M. J.* 28: 173, 1932.
4. Einsel, I. H., Adams, W. L., and Myers, V. C.: Aluminum Hydroxide in the Treatment of Peptic Ulcer, *Am. J. Digest. Dis. & Nutrition* 1: 513, 1934.
5. Adams, W. L., Einsel, I. H., and Myers, V. C.: Aluminum Hydroxide as Antacid in Peptic Ulcer, *Am. J. Digest. Dis. & Nutrition* 3: 112, 1936.
6. Kagan, S. R.: The Treatment of Gastric Ulcer and Hypochlorhydria, *Clin. Med.* 32: 229, 1925.
7. Gillermin, R.: Hyperchlorhydria et hydrate d'aluminium colloidal, *Rev. med. de la Suisse Rom.* 44: 243, 1924.
8. Surmont and Cauvin: *L'Echo Méd. du Nord.*, Oct. 10, 1925.
9. Woldman, E. E., and Rowland, V. C.: Continuous Acid Absorption by Aluminum Hydroxide Drip in the Treatment of Peptic Ulcer, *Rev. Gastroenterol.* 3: 27, 1936.
10. Woldman, E. E., and Rowland, V. C.: New Technique for the Continuous Control of Acidity in Peptic Ulcer by the Aluminum Hydroxide Drip, *Am. J. Digest. Dis. & Nutrition* 2: 733, 1936.
11. Jones, C. R., Jr.: Colloidal Aluminum Hydroxide in the Treatment of Peptic Ulcer, *Am. J. Digest. Dis. & Nutrition* 4: 99, 1937.
12. Mann, F. C., and Bollman, J. L.: Experimentally Produced Peptic Ulcers, *J. A. M. A.* 99: 1576, 1932.
13. Dragstedt, L. R.: The Surgical Treatment of Duodenal Ulcer, *Rev. Gastroenterol.* 5: 34, 1938.
14. Wilhelmj, C. M.: Test Meals and Methods of Gastric Analysis, *Am. J. Digest. Dis. & Nutrition* 4: 602, 1937.
15. Beazell, J. M., Schmidt, C. R., and Ivy, A. C.: The Effect of Aluminum Hydroxide Cream on Absorption From the Gastro-intestinal Tract, *Am. J. Digest. Dis.* 5: 164, 1938.
16. Ivy, A. C., Terry, L., Fauley, G. B., and Bradley, W. B.: The Effect of Administration of Aluminum Preparations on the Secretory Activity and Gastric Activity of the Normal Stomach, *Am. J. Digest. Dis. & Nutrition* 3: 879, 1937.
17. Meschan, I., and Quigley, J. P.: Spontaneous Motility of the Pyloric Sphincter and Adjacent Regions of the Gut in the Unanesthetized Dog, *Am. J. Physiol.* 121: 350, 1938.

# A REPORT OF THREE CASES OF TORULA INFECTION OF THE CENTRAL NERVOUS SYSTEM\*

R. GREGORY MAGRUDER, M.D., CHARLOTTESVILLE, VA.

THE first instances of invasion of the central nervous system by the yeast-like, budding organism, *Torula histolytica*, were reported in the German literature in 1905<sup>1</sup> and 1907.<sup>2</sup> Since that time the disease has been found to occur almost ubiquitously, and its various aspects have been well covered in the subsequent reports. More recently Levine<sup>3</sup> has collected and analyzed in detail a total of 58 well-authenticated cases and added two of his own. His careful notations describe in a concise and comprehensive manner the diagnosis and clinical course of this disease.† It has been shown<sup>3</sup> that torula infestation of the central nervous system may resemble tuberculous meningitis, meningitis (unqualified), encephalitis, tumor of the brain, and less frequently, abscess of the brain, dementia psychosis, and dementia paralytica.

Since October, 1935, three instances of torula involvement of the central nervous system have been observed at the University of Virginia Hospital. It is thought worth while to add these cases to those previously reported, making a total of 66 cases now in the literature.

## CASE REPORTS

CASE 1.—A 28 year-old colored washerwoman was admitted to the University of Virginia Hospital on October 7, 1935, complaining of headache and vomiting.

The family, past, and marital histories were irrelevant.

The onset of headache occurred seven weeks prior to her admission, and was noted at first in the occiput, later shifting to the frontal and left temporal areas. The pain was dull and more or less constant. Nausea and vomiting appeared early, and diplopia and transient blindness were noted. Visual acuity had become gradually and markedly diminished. Transient deafness had occurred in both ears, variably, but without tinnitus. Several weeks after the onset, she had had a sudden generalized convulsion, with complete loss of consciousness and involuntary discharges. Following this occurrence, the patient was placed in her local hospital where the following observations were recorded:

Malnutrition, a feeble and rapid pulse, hypotension (96/80). The optic disks showed definite choking, more marked on the right side. Knee jerks were absent. Albuminuria was marked (four plus). The spinal fluid was under increased pressure and clear; the globulin was slightly increased, and a cell count of 310, predominantly lymphocytes, was recorded. No organisms were noted. Lumbar puncture was repeated several days later, and the spinal fluid pressure was found to be 300 mm. of water. The cell count was now 280. Wassermann and Kahn tests were negative. Colloidal gold curve indicated only meningeal irritation. A diagnosis of intracranial tumor was made, and transference to the University of Virginia Hospital advised.

\*From the Department of Internal Medicine, University of Virginia Medical School, University, Va.

Received for publication, June 22, 1938.

†Since the initiation of this study, G. T. Caldwell in the Texas State J. Med. August, 1937; J. T. Cram et al. in the Am. J. Path. September, 1937, and J. H. Cudmore and J. R. Lisa in the Ann. Int. Med. March, 1938, have brought the number of hitherto reported cases to 63.

On admission to this hospital, general physical and neurologic examinations showed the following abnormalities: a malnourished colored girl; generalized tenderness over the head; ptosis of the right eyelid; weakness of the left lateral rectus and right internal rectus muscles; rotary nystagmus with upward and lateral gaze; definite choking of the optic disk, the right greater than the left; old retinal hemorrhages; gingivitis; several carious teeth; and blood pressure 90/68.

Routine laboratory studies of the blood, urine and stool were normal.

A tentative diagnosis of brain tumor was made.

On October 9, x-ray films of the skull showed calcium deposits in the pineal region and projecting over the foramen magnum, which were believed to represent calcification in a meningioma or glioma.

On October 10, three days after admission, neurologic examination showed the following changes: ptosis of both eyelids, particularly the right; diminished light reflex; inability to wrinkle the left forehead or close the left eye tightly; weakness of the left facial muscles; diminished biceps, triceps, periosteal and abdominal reflexes; absence of the left patellar reflex, both Achilles reflexes, and diminished right patellar reflex; and slight past pointing.

On October 14, one week after admission, an encephalogram was done and thought to be within the limits of normal. The initial spinal fluid pressure was over 550 mm. of water. The fluid was cloudy. Globulin was increased (three plus). A cell count of 794, with a slight predominance of polymorphonuclear cells, was recorded. Spinal fluid sugar was 44 mg., chlorides 620 mg., and total protein 152 mg. per 100 c.c., respectively. Wassermann test and colloidal gold curve were negative. A mouse was inoculated with the fluid and later autopsied. Smears and stains of various organs were negative for any organisms.

In view of the physical findings, the laboratory, and the x-ray studies, there was considerable controversy as to the diagnosis in this case, as so few of the findings were correlated. The patient continued to have headache and vomited at intervals. Since her hypotension was thought to contraindicate an exploratory craniotomy, it was deemed advisable to perform a decompression operation in an attempt to save her eyesight. On October 19, a right temporal decompression operation was done. No superficial evidence of tumor was noted. The dura appeared normal. Following this procedure, the patient's headache was relieved temporarily. A guinea pig was inoculated into the groin with spinal fluid taken at the time of the operation.

A neurological examination on October 27 showed the following changes: Weakness of movement of the eyes to the left; inability to converge; hippus reaction of the pupils to light; absence of accommodation; and greatly diminished tendon reflexes.

The encephalogram was repeated on October 31, but showed no appreciable change since the previous one.

On November 1, the patient became irrational, had delusions and hallucinations, and complained of severe headache. A distinct bulging of the decompression site was noticed. This was relieved to some extent by lumbar punctures at intervals, at which times the spinal fluid was always under increased pressure and there was a cell count varying from 884 to 1,330. A culture of the spinal fluid was positive for a gram-positive bacillus and a yeast, which were thought to be contaminants.

As the patient showed no improvement, it was decided to explore for brain tumor. On November 7, a right exploratory craniotomy was performed. Examination of the dura showed numerous areas of grayish exudate along the vessels on the surface of the brain as well as several small cysts. The brain was examined in several places, but no tumor was found. A segment of a cyst wall and some of the grayish exudate were sent to the pathological laboratory for diagnosis. After examination of the tissue, including a bacterial stain, the diagnosis was subacute torula meningitis. Following operation, the patient grew rapidly worse and died the same day, November 7, 1935. An autopsy was not performed.

On November 29, the guinea pig inoculated in the groin with spinal fluid on October 19 was autopsied. Organisms identified as torula were found in an abscessed gland.

*Comment* Case 1, on admission, presented a clinical picture resembling intracranial tumor. In view of the changing neurologic findings and the results of the laboratory and x-ray studies, the diagnosis was extremely problematical. Unsuspectingly, it had been made by culturing the yeastlike organism from the spinal fluid. Through the error of regarding it as a contaminant, this evidence was discarded. As a result, not until craniotomy had been done was the correct pathologic process determined.

Case 2—A 50 year old white farmer was admitted to the University Hospital on April 6, 1937, complaining of headache, weakness, and drowsiness.

The family, marital and past histories were irrelevant with the exception of two previous admissions to this hospital, the first having been in April 1917 at which time the diagnosis was diabetes mellitus and lymphatic leukemia. He had been readmitted to the hospital in June 1927, for regulation of his diabetes. X-ray therapy for the leukemia condition was not deemed necessary at that time. Since his last discharge from this hospital the patient had been in a poor state of health. He had had twelve x-ray treatments in another hospital the last treatment having been given about three months before his present admission. He had been adhering to his diabetic diet fairly well. The urine had been sugar free when examined by him at home.

About a week before admission the patient began to complain of a constant dull, diffuse headache. His local physician had prescribed medicine which had not given relief. Four days before admission, while driving an automobile home he had experienced a transient paralysis of the right arm and leg and the car had swerved off the road into a field, there was no injury. The next day he seemed drowsy and talked very little. He continued to complain of increasing headache and weakness, so was brought to the hospital for examination.

Physical examination showed the following abnormal findings: moderate drowsiness, moist skin, pale mucous membranes, bilateral pterygia, sclerosis of the fundal arteries, clouded atri, dental caries, gingivitis, postnasal discharge, slight stiffness of the neck, enlarged, discrete, and firm, bilateral, anterior and posterior cervical, axillary, inguinal, and epitrochlear glands, the largest being about 5 by 6 cm., slow but regular respiration, the rate being 11 per minute, several palpable masses in the abdomen thought to be enlarged lymph glands, diminished abdominal and cremasteric reflexes, absent knee jerks.

Laboratory studies were as follows: hemoglobin 71 per cent (Dare), erythrocytes 4,500,000, leucocytes 20,000. A differential count of a stained blood smear showed 12 per cent degenerated cells, 1 per cent myelocytes, 4 per cent band cells, 12.5 per cent segmented polymorphonuclear cells, 2 per cent large lymphocytes, 70 per cent small lymphocytes and 0.5 per cent histophiles. Blood Wassermann and Kahn tests were negative. The urine on admission reduced Fehling's solution with one drop, acetone and diacetic acid reactions were strongly positive. Microscopic examination showed 8 to 10 white blood cells per high power field and a few hyaline casts.

The admission temperature was 98° F and pulse 48. On the basis of the urinary findings, it was thought that the patient's condition was most probably due to diabetic acidosis. A specimen of blood was withdrawn immediately to determine the blood sugar and carbon dioxide combining power. Treatment for acidosis was instituted at once. The admission blood sugar was 190 mg per 100 cc and the carbon dioxide combining power was 54.9 volumes per cent. In view of these findings and the fact that the patient's urine became free of sugar, acetone, and diacetic acid after a few hours treatment, and there was no improvement in his lethargic state, it was thought advisable to do a lumbar puncture, which was performed on the night of admission. The initial pressure was 410 mm of water. The spinal fluid was very slightly cloudy and a questionable xanthochromic tinge was noted. A cell count of 100 was obtained with the polymorphonuclear cell and the lymphocyte counts being approximately equal. While making the cell count yeastlike budding organisms, which were thought to be toruli, were seen on the preparation. Culture of the spinal fluid was also reported positive for yeastlike organisms and later identified as *Torula histolytica*. The

spinal fluid chlorides were 590 mg., total protein 100 mg., and sugar 30 mg. per 100 cc., respectively. The spinal fluid Wassermann and colloidal gold curve were negative. A diagnosis of torula meningitis was made.

On April 9, an x ray film of the chest showed several fairly well-defined areas of cotton like infiltration in the left base. There was some increase in fibrosis in the lung fields on the right, particularly in the right base. There was a small, indefinite area of infiltration, about 1 cm. in length, superimposing the third interspace, anteriorly, on the right. On the same day a microscopic examination of the spinal fluid was again positive for yeast-like budding organisms. A blood culture and a culture of the urine were negative for torula. On the following day a left axillary lymph gland was biopsied. The tissue diagnosis was lymphatic leucemia.

During his stay in the hospital, the patient had a slight, daily rise of temperature. He took food and fluids very poorly. Control of the blood sugar level was difficult. He complained of a constant, severe, diffuse headache. Lumbar punctures were done once or twice daily. He was given sodium iodide intravenously and large doses of potassium iodide orally. On April 12, he appeared very much worse. There was much more stiffness of the neck. He appeared extremely restless, but at the same time drowsy. The following day, April 13, he was quite irrational, and voided in bed. His neck and back were very stiff, and he complained of pain in both legs. At 7 p.m. he became more restless and tossed about in bed, moving all extremities. Fifteen minutes later, flaccid paralysis of the right arm and leg was noted. Respiration became deep and labored. All tendon reflexes were absent. The tongue deviated to the left. A few minutes later the patient died.

Summary of post mortem findings: Lymphoid leucemia of unusual type with generalized involvement of lymph nodes, bone marrow, spleen and liver; torula infection involving lungs, cerebrospinal meninges, spleen, and prostate; focal hyaline islands of Langerhans; healed tuberculosis lungs and tracheobronchial lymph nodes; pleural adhesions; arteriosclerosis aorta and large branches; small hemangioma, liver.

*Comment:* This was a case of torulosis in a patient with diabetes mellitus and lymphatic leucemia. The diagnosis was made by the unsuspecting finding of yeastlike budding organisms on microscopic examination of the spinal fluid. These were identified by further laboratory procedures as *Torula histolytica*.

CASE 3.—A 22 year old colored housewife was admitted to the University of Virginia Hospital on April 23, 1937, complaining of pain in the head and neck.

The family, past, and marital histories were not of interest.

Since January, 1937, the patient had had an intermittent frontal headache, which had increased in severity during the last five weeks and had been accompanied by pain in the neck. Earache was present at times, and tinnitus more or less constantly. Her appetite was poor and vomiting had occurred frequently. Visual acuity had diminished. Photophobia was noted, and "blind staggers" had been experienced. More recently, sedatives were required at night for sleep. The day before admission the right hand and arm felt numb, but there was no loss of use. During her present illness, the patient had attempted to continue her work when she felt well enough. Several hours before admission, she had been found lying on her bed in an irrational condition. Her local physician had advised her entering the hospital.

General physical and neurologic examinations revealed the following abnormal findings: an irrational colored female; definite choking of the optic disks, the right greater than the left; a large amount of exudate and several recent hemorrhages; dental caries; diseased tonsils; slight deviation of the tongue to the left; slight stiffness of the neck; left biceps reflex greater than the right; sluggish patellar reflexes and absent Achilles tendon reflexes.

Routine blood studies were normal. Urinalysis showed only an occasional granular cast. There was a slightly positive benzidine test on a stool examination. X-rays of the skull showed no abnormalities.

On admission a tentative diagnosis of brain tumor was made. During her stay in the hospital, the patient ran a slightly elevated temperature. On April 29, a ventriculogram was made. X-ray films showed the ventricular system to be fairly well filled with air, and no abnormalities were demonstrated. In view of this report and because of the continuation of the severe headache, a lumbar puncture was done on May 1 in an attempt to relieve the patient and study the spinal fluid. The initial pressure was 450 mm of water. The Queckenstedt test was normal, the fluid was xanthochromic. The total protein was 75 mg, chlorides 650 mg, and sugar 24 mg per 100 cc, respectively. There was a cell count of 451, with a predominance of lymphocytes. The spinal fluid Wassermann and colloidal gold curve were negative. A Gram stain of the spinal fluid sediment showed yeastlike organisms, which were cultured and identified as *torula*.

During the remainder of her stay in the hospital, the patient continued to have severe headache, which was relieved temporarily by repeated drainage of the spinal fluid. The spinal fluid cell count on May 6 was 185, with a predominance of lymphocytes. Yeastlike organisms were again found. She was discharged from the hospital on May 8, 1937 having refused further treatment.

The patient reported back to the clinic January 28, 1938. She stated that since her discharge from the hospital her health had been good, except for transient pains in the vertex of her head and back of her neck. These pains occurred about once a week usually lasted five or ten minutes and were accompanied by nausea but no vomiting. Lumbar puncture was done in the clinic. The initial pressure was 120 mm of water. The spinal fluid dynamics were normal. The fluid was very slightly cloudy. There was a cell count of 120, with a predominance of lymphocytes. Colloidal gold curve was 5555555510. Spinal fluid protein was 100 mg, chlorides 630 mg, and sugar 21 mg per 100 cc respectively. Yeastlike organisms were again found on microscopic examination and were also cultured from the spinal fluid and identified as *torula*.

**Comment.** Case 3 is one of *torula* infection of the central nervous system of one year's duration. There has been no treatment since discharge from the hospital on May 8, 1937. The patient is still doing her daily work with little inconvenience from the infection. Levine,<sup>4</sup> in his article, found the average length of the disease to be five or six months. The longest case on record lived five and one half years.

#### REFERENCES

- 1 von Hansemann. Ueber eine bisher nicht beobachtete Gehirnkrankung durch Hefen, Verhandl d deutsch path. Gesellsch 9 21, 1905
- 2 Turk, W. Ein Fall von Hefeninfektion (Saccharomycose) der Meningen, Arch f Klin Med 90 335, 1907
- 3 Levine, E. A. *Torula* Infection of the Central Nervous System, Arch Int Med 59 67, 1937

## A NOTE ON THE STABILITY OF PICROTOXIN SOLUTIONS\*

PHOEBE J. CRITTENDEN, PH.D., WASHINGTON, D. C.

IN LIEU of the experiments of Tatum and his associates<sup>1-3</sup> on the use of analeptics in barbiturate and other hypnotic poisonings, it seemed likely that picrotoxin would become more important as a therapeutic agent. Hence a knowledge of its stability in solution when kept over long periods of time might be of value.

Since our experiments were started, Koppányi and his associates<sup>4-6</sup> have again emphasized the importance of picrotoxin as an antidote in barbiturate poisoning.

Arnett<sup>7</sup> was the first to use it in a human case. Recently others<sup>8-10</sup> have used it with equal success.

### PROCEDURE

In December, 1935, 0.05 per cent aqueous solutions of two different picrotoxin preparations A and B were made. Both solutions, after testing for toxicity as described below, were divided into three portions and kept in pyrex flasks; one was kept at 5° C.; a second at room temperature; and the third at 37° C.

These solutions were tested for their toxicity at various intervals, varying from one week to six months. White male rats, weighing about 150 gm., were injected in the femoral vein. The time of onset of convulsions, the severity of the convulsions, the time of death, and the mortality rate were noted.

### RESULTS

Both of the original solutions killed three out of four rats with a dosage of 3 mg. per kg. This dosage was used in most of the subsequent experiments. The solutions kept in the refrigerator and at room temperature varied in the number of rats killed at various times, but appeared relatively stable after two years. The solutions kept in the incubator, however, remained stable for about four to six months. After a year, they failed to kill in the dosage of 3 mg. per kg. The severity of the convulsions also decreased. After two and one-half years, these solutions were tested by increasing the dosage to 4 mg., 5 mg., and 6 mg. per kg. It was found that these solutions (37° C.) had lost about 60 per cent of their original potency because 5 mg. per kg. killed five out of six rats.

\*From the Department of Pharmacology and Therapeutics, School of Medicine, the George Washington University, Washington, D. C.

Received for publication, June 22, 1938.

## DISCUSSION

Our initial toxic dose agrees well with that of Haas<sup>11</sup> He found that 2.8 mg per kg injected subcutaneously killed two out of five rats and that 3.2 mg per kg killed four out of six rats.

Our results indicate that picrotoxin solutions are relatively stable for four to six months after preparation when kept in pyrex flasks at temperatures ranging from 5° C to 37° C When kept in the refrigerator and at room temperature, they appear to keep fairly well for about two years The solutions kept in the incubator are less stable If picrotoxin solutions are to be used in warm climates, they should be kept in a cool place

No attempt was made to keep the solutions sterile Those kept in the refrigerator showed a growth of mold in about nine months This did not appear to affect the potency

## SUMMARY

Solutions of picrotoxin A and B kept at temperatures varying from 5° C to 37° C appear to be stable for four to six months Those kept in the refrigerator and at room temperature are relatively stable for two years, while those kept in the incubator lose about 60 per cent of their potency in two and a half years

## REFERENCES

- 1 Tatum, A. L., Fitch, R. H., and Maloney, A. H. Picrotoxin in Barbituric Acid Poisoning, *J Pharmacol & Exper Therap* 41: 465, 1931
- 2 Maloney, A. H., and Tatum, A. L. Picrotoxin as an Antidote in Acute Poisoning by the Longer Acting Barbiturates, *J Pharmacol & Exper Therap* 44: 337, 1932
- 3 Maloney, A. H. A Comparative Study of the Antidotal Action of Picrotoxin, Strychnine and Cocaine in Acute Intoxication by the Barbiturates, *J Pharmacol & Exper Therap* 49: 133, 1933
- 4 Linegar, C. R., Dille, J. M., and Koppányi, T. Antidotal Action of Picrotoxin in Extreme Case of Experimental Poisoning, *Proc Soc Exper Biol & Med* 33: 396, 1935
- 5 Linegar, C. R., Dille, J. M., and Koppányi, T. The Nature of the Barbiturate Picrotoxin Antagonism, *Science* 82: 376, 1935
- 6 Koppányi, T., Linegar, C. R., and Dille, J. M. Studies on Barbiturates, XIX. Analysis of the Barbiturate Picrotoxin Antagonism, *J Pharmacol & Exper Therap* 58: 199, 1936
- 7 Arnett, J. H. Ephedrine and Picrotoxin Used Successfully in Amytal Poisoning, *J A M A* 100: 1593, 1933
- 8 Maloney, A. H. The Protective Action of Picrotoxin in Prolonged Surgical Anesthesia with Amytal, *Am J Surg* 34: 571, 1936
- 9 Murphy, W. S., Connerty, H. V., Connolly, A. J., and Koppányi, T. Barbiturate Poisoning Treated With Picrotoxin, *J Lab & Clin Med* 22: 350, 1937
- 10 Khne, E. M., Begg, E., and Whitney, H. A. K. Picrotoxin in the Treatment of Barbiturate Poisoning, *J A M A* 109: 328, 1937
- 11 Haas, H. T. A. Über den Antagonismus der Narkotica gegen die Analeptica, Coramin und Picrotoxin, *Arch f exper Path u Pharmacol* 184: 468, 1937



## THE HEMOGLOBIN CONTENT OF HUMAN BLOOD\*

VICTOR C. MYERS, PH.D., D.SC., AND HELEN M. EDDY, M.S.  
CLEVELAND, OHIO

THE need for more accurate data on the hemoglobin content of normal human blood, and the desirability of recording such observations in grams per 100 c.c., or actual per cent, were emphasized in this JOURNAL by one of us<sup>1</sup> a number of years ago (1921), the concluding statement being: "It is suggested that the time honored hemoglobin test would yield results of much greater value if more attention were given to accurate standards and methods, and the results then computed to give the actual percentage of hemoglobin rather than the relation to an indefinite normal in parts per hundred." Although clinical workers were rather slow to adjust themselves to this newer point of view, the hope expressed would appear to have been fairly completely realized at the present time. The statement was also made: "In the past one of the greatest difficulties in the way of making an accurate colorimetric hemoglobin estimation has been to secure a correct standard. This is now in a fair way to solution owing to the development by Van Slyke of a simple method of determining the oxygen capacity of the blood." It turns out that the oxygen capacity method of Van Slyke<sup>2</sup> has been the chief factor in enabling workers in various parts of the world to collect accurate data on the hemoglobin content of blood. The Van Slyke and Neill<sup>3</sup> method has been employed in practically all recent work, either directly, or as the method of reference in checking the standards. For this reason all observations are now on a common and comparable basis.

The chief explanation why the older hemoglobin observations were not on a comparable basis was due to the fact that the different instruments available generally took 100 as the normal, but did not agree on the equivalent of this 100 in gm. per 100 c.c. of blood. For example, the Dare instrument used 13.77 gm. of hemoglobin per 100 c.c. of blood as its standard of 100, while Sahli at the other extreme took 17.2 gm. of hemoglobin as its equivalent of 100 per cent.<sup>4</sup> Strangely enough it would appear that the two extremes were the standards most used. The results were further complicated by the fact that the methods were not very exact, the standards often deteriorated and there was no simple method of checking them. With the use of the Van Slyke oxygen capacity method, either to determine the hemoglobin directly or to check the standard for colorimetric use, and the employment of a precise colorimeter, as in other colorimetric analyses, the situation has been completely changed.

\*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

The data presented in this paper are taken from the thesis submitted by Helen M. Eddy to the Graduate School of Western Reserve University, June, 1938, in partial fulfillment of the requirement for the degree of Master of Science.

Received for publication, December 30, 1938.

Since exact and relatively simple methods of hemoglobin estimation have become available, observations have been made in all parts of the world on the concentration of hemoglobin in the blood of the normal human adult in an effort to define more exactly the normal. In following this work it seemed to us at first that there might be some differences due to geographic location and that it might be helpful to make a series of observations in Cleveland. We also wished to ascertain the comparative accuracy of three different micro methods on samples of capillary blood.

Since our study was undertaken, a considerable number of observations have been reported. These and our own studies have led us to the conclusion that normal adults in good nutrition, not residing at excessive altitudes above sea level show essentially the same concentration of hemoglobin in the blood with out difference due to race or geographic location. The only difference is that due to sex, which has long been recognized.

TABLE I

RECENT DATA ON THE HEMOGLOBIN CONTENT OF THE BLOOD OF YOUNG ADULT MALES

INVESTIGATORS	YEAR	NO OF SUBJECTS	GEOGRAPHIC LOCATION	RED BLOOD CELL COUNT	HEMOGLOBIN
				Millions	Gm per 100 cc
Garguilo	1936	45	Cordoba, Argentina	-	17.04
Wintrobe and Millers	1929	100	New Orleans	5.87	17.00
Brown and Briggs	1914	22	St. Louis	5.29	16.00
Sankaran and Rajagopals	1918	125	Madras, India	-	16.57
Heilmeyer and Hausold	1911	40	Leipzig, Germany	5.06	15.98
Jenkins and Donio	1913	116	England	-	15.85
Osgood <sup>11</sup>	1935	259	Portland, Ore.	5.42	15.84
Present Study	1938	111	Cleveland	-	15.83
Wintrobe <sup>12</sup>	1937	86	Baltimore	5.41	15.80
Osgood <sup>13</sup>	1926	137	Portland, Ore.	5.29	15.76
Foster and Johnson <sup>14</sup>	1931	115	New Orleans	5.26	15.63
Fildes and Witney <sup>15</sup>	1916	20	Saskatoon, Saskatchewan	5.32	15.55
Price Jones (Dill) <sup>16</sup>	1931	20	Boston	-	15.46
Gokhale, Gokhale, Maland <sup>17</sup> , Bilimoria <sup>18</sup>	1937	121	Bombay, India	5.11	15.37
Haden <sup>19</sup>	1922	30	Kansas City	4.95	15.34
Ornato	1930	82	Buenos Aires	-	15.30
Berring, Nielsen and Nielsen <sup>20</sup>	1935	10	Copenhagen, Denmark	-	15.26
Walters <sup>21</sup>	1934	100	Kansas City	4.84	15.12
Seels, Levine, and Fabrian <sup>22</sup>	1935	200	Omaha	4.88	14.96
Napier and Dhillon <sup>23</sup>	1935	50	Cuttack, India	5.36	14.77
Lie Jones <sup>24</sup>	1931	100	London, England	5.43	14.55

Observations on some 3,000 adults are summarized in Tables I and II. Most of the observations were made on young adults, chiefly medical students and nurses. Some investigators carried out studies on groups with different social status but in such cases only student groups were included in the tables. It has not seemed necessary or helpful for the present presentation to discuss each individual study separately. Some workers compared several different methods, but the average of one set of determinations has been used for the tables. No effort has been made to indicate the method used to obtain the average hemoglobin figure given in the tables, since it is not believed that the differences ob-

served are due primarily to either the methods employed or to geographic location. It will be noted that in several instances different workers in the same city obtained quite different results. The interesting fact is that the maximum difference is only 15 per cent. It will be observed in Table I that for the adult male the maximum average is 17.04 gm. hemoglobin and the minimum figure is 14.55 gm. Both of these values lie between the normal figures originally taken for 100 in the Sahli and Dare instruments, respectively.

TABLE II

RECENT DATA ON THE HEMOGLOBIN CONTENT OF THE BLOOD OF YOUNG ADULT FEMALES

INVESTIGATORS	YEAR	NO. OF SUBJECTS	GEOGRAPHIC LOCATION	RED CELL COUNT	HEMO GLOBIN
				Millions	Gm. per 100 c.c.
Broun and Briggs <sup>7</sup>	1934	13	St. Louis	4.78	14.70
Heilmeyer and Hausold <sup>9</sup>	1936	28	Jena, Germany	4.55	14.69
Wintrobe <sup>12</sup>	1933	101	Baltimore	4.80	14.05
Osgood <sup>11</sup>	1935	152	Portland, Ore.	4.83	13.91
Jenkins and Don <sup>10</sup>	1933	116	England	-----	13.80
Wintrobe <sup>24</sup>	1930	50	New Orleans	4.93	13.76
Sankaran and Rajagopal <sup>8</sup>	1938	62	Madras, India	-----	13.73
Gargiulo <sup>5</sup>	1936	83	San Luis, Argentina	-----	13.73
Gargiulo <sup>5</sup>	1936	20	Cordoba, Argentina	-----	13.70
Osgood and Haskins <sup>25</sup>	1927	100	Portland, Ore.	4.80	13.69
Price-Jones <sup>16</sup>	1931	100	London, England	5.01	13.60
Orias <sup>19</sup>	1930	22	Buenos Aires	-----	13.38
Haden <sup>18</sup>	1922-33	30	Kansas City	4.38	13.37
Present Study	1938	48	Cleveland	-----	13.10
Gargiulo <sup>5</sup>	1936	46	Mercedes, Argentina	-----	13.09
Sokhey, Gokhale, Maland- kar, Billimoria <sup>26</sup>	1938	101	Bombay, India	4.47	12.99
Sachs, Levine, and Fabian <sup>22</sup>	1935	100	Omaha	4.51	12.96

If one selects the mean of the values recorded in the tables for both the adult male and female, the figures will be 15.8 gm. for the male and 13.8 gm. for the female, the same figures as those obtained by Osgood<sup>11</sup> in his own series. It should be noted that Wintrobe<sup>22</sup> arrived at essentially the same figures in his summary of reported values, viz., 15.9 gm. for the adult male and 13.9 gm. for the female. It is well recognized from Williamson's classic observations that the hemoglobin tapers off slightly with advancing years, and the more nearly round number figures of 15.5 gm. for men and 13.5 gm. for women should approximate the average for the last two or three decades of life, the variation from the normal average for either sex being about  $\pm 1.5$  gm.

It has long been recognized that the hemoglobin level at birth is very high, 20 to 22 gm., probably due to the low oxygen tension existing during fetal life, but after birth the level sinks quite rapidly, until between the second and third months it is down to 10 gm., after which it rises to 12 gm. between the fifth and sixth months, where it remains during childhood.

As already indicated the authors doubt the wisdom of continuing to employ the old clinical standard of 100 as the normal for hemoglobin. However, for those who still prefer this method in clinical work, it is suggested that the use of the arbitrary standard of 15.0 gm. hemoglobin per 100 c.c. of blood as the equivalent of 100 per cent hemoglobin might be found a more convenient figure

than any other for the reasons enumerated below (1) 150 gm is as near a round number figure as it is possible to select, and only slightly below the normal given by Haden<sup>18</sup> (2) If one takes the normal for adult males as 150 gm, for adult females as 135 gm, and children as 120 gm, then the 100 per cent standard for males would read 90 per cent for females and 80 per cent for children figures which may be easily remembered and interpreted

# IRON AND HEMOGLOBIN ESTIMATIONS

A number of different workers have utilized the estimation of total iron as a means of hemoglobin estimation apparently with good results. This is true of several of the investigators whose observations are recorded in Tables I and II although the only figures given in the tables which were obtained by the iron method are those of Sachs, Levine and Palmer. Klumpp<sup>26</sup> has objected to this use of total iron, since in agreement with Barkan he has found in his series of experiments an average of 53 per cent of total iron uncombined with active hemoglobin, i.e., as nonhemoglobin iron. He states: 'These results indicate that attempts to arrive at values for hemoglobin by determination of total blood iron or vice versa, are subject to an average error of 53 per cent with a standard deviation of 39 per cent, and a possible error as great as 178 per cent.' In a later publication Klumpp<sup>28</sup> states: 'Peters (R. A.) working in Barett's Laboratory demonstrated in 1912 that hemoglobin combines with one molecule of oxygen per atom of iron. With this fundamental relationship as a basis it becomes possible to calculate accurately values for hemoglobin iron from the oxygen combining capacity of the blood.'

Theoretically, as Klumpp<sup>26</sup> states, there are three forms of iron which need to be considered in blood, viz., hemoglobin iron, nonhemoglobin iron, and plasma (or serum) iron. The inorganic iron of the serum as shown by Bing, Hanzal, and Mers<sup>29</sup> amounts to between 0.1 and 0.2 mg iron per 100 cc. Since the normal iron content of whole blood is close to 50 mg this serum (or plasma) iron would introduce an error of less than 0.5 per cent quite outside the limits of error of the methods ordinarily used for hemoglobin estimation.

In a series of some fifty samples of human blood Klumpp<sup>27</sup> found that the iron calculated on the basis of oxygen capacity, averaged 53 per cent less than that determined directly. Despite the care which Klumpp undoubtedly exercised in his determinations, his findings in this regard are in disagreement with those of a number of workers who have obtained excellent agreement between hemoglobin calculated from oxygen capacity determinations and from total iron or from protein iron. Among these might be mentioned Landsay, Rice, and Selinger,<sup>30</sup> Kennedy,<sup>31</sup> Helmer and Emerson,<sup>32</sup> Haden,<sup>33</sup> Brown and Briggs,<sup>7</sup> and Johnson and Hanke,<sup>34</sup> whose observations are summarized in Table III. Andes and Northrup,<sup>35</sup> and Breuer and Miltzer,<sup>36</sup> in observations recently published, have also reported excellent agreement between figures for hemoglobin calculated from total iron and those obtained by methods of hemoglobin estimation other than oxygen capacity.

In general, it will be noted that the observations for hemoglobin calculated from the total iron tend to be slightly high while those calculated from the

protein iron tend to be slightly low. The agreement may be said to be very good, however, and within the limits of ordinary experimental error. Kennedy,<sup>31</sup> and Johnson and Hanke<sup>34</sup> took great care with their iron determinations, the latter employing a very accurate volumetric method which they state did not have a maximum error greater than 0.5 per cent. As will be noted, Kennedy,<sup>31</sup> and Johnson and Hanke<sup>34</sup> obtained a plus difference for hemoglobin calculated from total iron in comparison with determinations calculated from the oxygen capacity of +0.5 and +0.7 per cent, respectively. When it is considered that their figures for iron included the plasma iron, the agreement is excellent. From the observations recorded in Table III it would appear legitimate to employ figures for total iron as the basis of hemoglobin estimations.

TABLE III

COMPARISON OF AVERAGE HEMOGLOBIN ESTIMATIONS CALCULATED FROM DETERMINATIONS OF OXYGEN CAPACITY, TOTAL IRON, AND PROTEIN IRON

INVESTIGATORS	SEX IF NOTED	NO. OF BLOODS	HEMOGLOBIN VALUES CALCULATED FROM				
			O <sub>2</sub> CAPACITY	TOTAL Fe	DIF- FERENCE FROM O <sub>2</sub> CAPACITY	PROTEIN Fe	DIF- FERENCE FROM O <sub>2</sub> CAPACITY
Lindsay, Rice, and Selinger (1926)		7	Gm. 13.33	Gm. 13.40	Pcr cent +0.5		
Kennedy (1927)		8	16.50	16.58	+0.5		
Helmer and Emerson (1934)	M	10	15.30	15.57	+1.8	15.15	-1.0
Helmer and Emerson (1934)	F	10	13.21	13.66	+3.4	13.43	+1.7
Haden (1934)		21	12.18	12.15	-0.25		
Brown and Briggs (1934)	M	23	16.76			16.53	-1.4
Brown and Briggs (1934)	F	13	14.15			13.82	-2.3
Johnson and Hanke (1936)		6	9.69	9.76	+0.7		

As a result of the work of Hüfner and others, it has been customary for many years to assume that the oxygen capacity of 1 gm. of hemoglobin was 1.34 c.c., and that the iron content of hemoglobin was 0.335 per cent. Recently, Morrison and Hisey<sup>37</sup> have made some very careful analyses which suggest that these values are slightly low. Their analyses indicate a minimum oxygen (and carbon monoxide) capacity of 1.36 c.c. per gram of hemoglobin and an iron content of 0.34 per cent. The ratio between these two figures, however, is the same as between the older values, so that their use would not change the comparative results for hemoglobin obtained from figures for oxygen capacity and iron content. In view of Klumpp's statement<sup>25</sup> it is interesting to note that Morrison and Hisey<sup>37</sup> concluded from 17 analyses of human hemoglobin that: "The average value of  $0.400 \pm 0.005$  c.c. compares with the theoretical value of 0.401 c.c. which assumes a ratio of 1 atom of iron to 1 molecule of carbon monoxide."

#### PRESENT OBSERVATIONS

The present study was undertaken, first, to obtain a series of observations on a fairly large group of healthy young adults residing in this locality, and secondly, to ascertain the comparative accuracy of three different micromethods on capillary blood. The three methods employed were the acid hematin method,<sup>22</sup>

using a Newcomer glass disk calibrated with the oxygen capacity method of Van Slyke, the non method of Hanzal,<sup>39</sup> and the benzidine method of Bung and Baker,<sup>40</sup> the last two methods having been developed in this laboratory.

*Subjects*—The male subjects were made up of students in the first year medical and dental classes in biochemistry with the addition of a few graduate students while the female subjects for the most part were undergraduate students in physiology in Flora Stone Mather College. All the subjects had passed satisfactory health examinations.

*Methods*—The blood was obtained by finger puncture after carefully washing with alcohol, with the aid of a 25 gauge syringe needle. The first drop was wiped away before any blood was drawn into the pipettes. At no time was the so called practice of "milking" the finger used. Blood was drawn up into calibrated capillary pipettes of 0.025 and 0.05 cc capacity similar to those employed for the Polm<sup>41</sup> microblood sugar estimation.

*Acid Hematin Method*—The Newcomer acid hematin method was carried out with 0.025 cc of blood exactly as described by Myers<sup>42</sup> employing a Klett biocolorimeter. The calibration of the Newcomer disk, with the aid of blood standardized with the Van Slyke oxygen capacity determination gave it a strength of 0.0314 per cent hemoglobin.

*Hanzal Iron Method*—As described by Hanzal<sup>39</sup> the thioglycolic acid method for non required 0.2 cc of blood. The method has been modified slightly to permit its use with 0.05 cc of blood. The following technique has been employed. 0.05 cc of blood is introduced into a Pyrex test tube (20 by 180 mm) calibrated at 5 cc, to which 1 cc of distilled water has been added. The blood is expelled slowly into the bottom of the test tube which is held in an inclined position. If this is done carefully, the blood will not mix with the water at the surface, and the water may be drawn up to the mark in the pipette several times to rinse out any blood which may cling to the sides of the pipette. One half cubic centimeter of 50 per cent sulfuric acid is added to the diluted blood. The digestion mixture is then heated with care over a microburner. As soon as the water has evaporated, the heating may be more vigorous. After the blood has charred, it is allowed to cool for about forty five seconds. Four drops of 30 per cent hydrogen peroxide (Merck's superoxol) are then added drop by drop, with heating between each addition, and the digestion mixture heated for about three to four minutes after it becomes clear. While the solution is still warm it may have a very faint yellow color, but on cooling it should be clear. If necessary an additional drop of hydrogen peroxide can be added and the procedure repeated until the solution becomes clear. One cubic centimeter of distilled water is then introduced into the test tube followed by one drop of thioglycolic acid. Concentrated ammonium hydroxide is then run in from a burette until the first permanent color appears (pH 8 to 10). Distilled water is added to make the volume up to 5 cc.

The standard non solution is prepared by dissolving 0.500 gm of analytical iron wire in 10 per cent sulfuric acid. Three cubic centimeters of concentrated nitric acid are added to oxidize the non to the ferric state, and the solution is then diluted to 500 cc (1 cc = 1 mg non).

The standard which is employed is made up daily from the concentrated standard iron solution by diluting 1.0 c.c. of the concentrated standard to 40 c.c. with distilled water. One cubic centimeter of the diluted standard is measured into a test tube calibrated at 5 c.c. It is then treated with one drop of thioglycolic acid, made alkaline with concentrated ammonium hydroxide, and made up to volume with distilled water.

Both standard and unknown are mixed by lateral shaking to insure complete development of color, and immediately compared in a colorimeter, with the standard set at 20 mm. If the color seems to be fading, as is sometimes the case, it may be restored by removing the solution from the colorimeter cup, shaking thoroughly, and rereading.

The reading of the standard, divided by the reading of the unknown, times 50 will give the milligrams of iron per 100 c.c. of blood. In calculating the per cent of hemoglobin the milligrams of iron were divided by 0.335, the figure which was taken as the per cent of iron in hemoglobin.

*Bing and Baker Benzidine Method.*—The determination was carried out exactly as described by Bing and Baker<sup>40</sup> (except that they used 0.005 c.c. of blood instead of 0.025 c.c.), which, briefly, is as follows: 0.025 c.c. of blood is obtained by finger puncture and discharged into a flask containing 50 c.c. of distilled water, the pipette being thoroughly rinsed. One cubic centimeter of the thoroughly mixed diluted blood is introduced into a test tube which already contains 2 c.c. of the benzidine reagent. Considerable caution should be observed to prevent any portion of the diluted blood touching the sides of the test tube. One cubic centimeter of 0.6 per cent hydrogen peroxide is then added to the blood benzidine mixture. One cubic centimeter of the diluted blood standard containing 0.05 mg. hemoglobin (prepared from blood standardized with the Van Slyke and Neill<sup>3</sup> oxygen capacity method) is treated in exactly the same manner. The tubes are allowed to stand for at least two hours for the full purple color to develop, diluted to 25 c.c. with 20 per cent acetic acid, stoppered with paraffined corks, inverted, and allowed to stand at least eight minutes before comparison in the colorimeter with the standard set at 10 mm. With the simplified calculation, 100 divided by the reading of the unknown in millimeters gives the grams of hemoglobin per 100 c.c. of blood.

The observations on our series of 111 males and 48 females are presented in summary form in Table IV. The individual observations are not presented since it was not felt that they would furnish sufficient added information to warrant the tabular space required. In general, the agreement between the individual determinations was excellent, although occasionally the deviations were greater than probably would have been the case if larger amounts of blood had been employed. The agreement between the averages for the three groups appears to be satisfactory.

The acid hematin method gave slightly the highest results and the benzidine method slightly the lowest results. Since the acid hematin method may be considered a standard method, it is of interest to note the percentage deviation which the two other methods show. Calculations made from the averages given

in Table IV show that in comparison with the acid hematin observations, the hemoglobin values calculated from the total iron are 0.3 per cent low in the male series and 1.1 per cent low in the female series while the results obtained with the benzidine method were 1.1 per cent low for the males and 2.0 per cent low for the females. However, if the data from the whole series of 159 subjects, including both males and females, are averaged, and comparison made on the basis of hemoglobin value calculated from the total iron the agreement appears even better. The average figure obtained with the acid hematin method is 0.5 per cent higher than that calculated from total iron while that obtained with the benzidine method is 0.8 per cent lower. Although we do not wish to present the observations obtained with these micromethods as proof that accurate figures for hemoglobin can be calculated from figures for total iron, still they do support this conclusion, and the deviations observed agree very well with observations from the literature recorded in Table III.

TABLE IV

HEMOGLOBIN CONTENT OF CAPILLARY BLOOD OF YOUNG ADULTS SIMULTANEOUSLY DETERMINED BY THREE MICROMETHODS

SEX	NO OF SUBJECTS		AGE	ACID HEMATIN*	IRON CONTENT	BENZIDINE
				Gm	Gm	Gm
Male	111	Maximum	30	17.69	17.15	17.86
		Minimum	19	13.80	13.69	13.33
		Average	23	15.83	15.78	15.66
Female	48	Maximum	20	15.82	15.27	14.71
		Minimum	17	11.63	11.40	11.37
		Average	20	13.10	12.93	12.84

\*Eighty per cent of the observations made with the acid hematin method fell between 14.4 and 16.8 gm per 100 cc in the males and between 12.4 and 14.0 gm in the females.

The average hemoglobin value obtained for the 111 male subjects, with an average age of 23 years, was 15.8 gm per 100 cc of blood, a figure identical with the mean of the figures given in Table I, and of the rather large series of observations (259) made by Osgood. This would not appear to require further comment.

In the case of the 48 females, with an average age of 20 years, the average hemoglobin value was 13.0 gm per 100 cc of blood, a figure definitely below the mean of Table II, namely, 13.8 gm per 100 cc of blood, which was also the value obtained by Osgood for 152 subjects. This would indicate that for some reason not readily apparent the women in our series had slightly subnormal hemoglobin values. This observation is receiving further attention.

Considering the small quantities of blood employed for each of the three determinations, it is believed that the agreement between the three methods may be considered good, about as good as could be expected with three entirely different colorimetric micromethods.

The question has sometimes been raised as to whether or not entirely satisfactory results for hemoglobin can be obtained on capillary blood secured by finger puncture. Since the average of our results on 111 adult male subjects



agrees exactly with the mean of the recent literature observations given in Table I, this would appear to support the reliability of observations made on finger blood

#### SUMMARY AND CONCLUSIONS

Attention has again been called to the desirability of recording hemoglobin values in grams per one hundred cubic centimeters of blood, as is done with other chemical blood determinations

The average hemoglobin value obtained on 111 normal young adult males residing in Cleveland was 15.8 gm per 100 cc of blood, a figure in exact agreement with the mean of the values obtained throughout the world

The average hemoglobin value obtained on 48 normal young adult females residing in Cleveland was 13.0 gm per 100 cc, a figure definitely below 13.8 gm, the mean of observations made throughout the world. This matter is receiving further attention

The results obtained with three micromethods, two of which employed 0.025 cc and one 0.050 cc of capillary blood, showed good agreement, considering the small amounts of blood employed. Comparing the averages of the results obtained on the 159 subjects, the Newcomer acid hematin method gave a hemoglobin value 0.5 per cent higher than that obtained with the Hanzl iron method, while the average value obtained with the Bing and Baker benzidine method was 0.8 per cent lower

It appears legitimate to calculate hemoglobin from figures for total blood iron.

#### REFERENCES

1. Myers, V. C. Editorial. The Hemoglobin Content of the Blood, *J. Lab & Clin Med* 6: 648, 1921
2. Van Slyke, D. D. Gasometric Determination of the Oxygen and Hemoglobin of Blood. *J. Biol Chem* 33: 127, 1918
3. Van Slyke, D. D., and Neil, J. M. The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, *J. Biol Chem* 61: 523, 1924
4. Queries and Minor Notes. Relative Values of Dare, Sahli, von Fleischl, Tallqvist and Oliver Hemoglobinometers. Standardizing Hemoglobinometers, *J. A. M. A.* 87: 1323, 1926, 88: 343, 1927
5. Garguilo, A. Hemoglobina sanguínea normal en personas de las provincias de Córdoba y San Luis (R. Argentina), *Rev. Soc. argent. de biol.* 12: 156, 1936
6. Wintrobe, M. M., and Miller, M. W. Normal Blood Determinations in the South, *Arch. Int. Med.* 43: 96, 1929; Wintrobe, M. M. Hemoglobin Standards in Normal Men, *Proc. Soc. Exper. Biol. & Med.* 26: 848, 1929
7. Brown, G. O., and Briggs, A. P. Hemoglobin and Blood Count Relations as Determined by Iron and Oxygen Capacity Methods, *J. Lab. & Clin. Med.* 19: 886, 1934
8. Sankaran, G., and Rajagopal, K. Hematological Investigations in South India. Part I. The Estimation of Hemoglobin, *Indian J. Med. Research* 25: 141, 1938
9. Heilmeyer, L., and Hausold, I. Der Hämoglobingehalt gesunder Männer und Frauen eine Studie zur Definition des relativen Massensystems der Hb Bestimmung und zur Aufstellung des normalen Farbeindex. 2. Mitteilung zur Hämometerstandardisierung, *Deutsches Arch. f. klin. Med.* 179: 94, 1936
10. Jenkins, C. E., and Don, C. S. D. The Haemoglobin Concentration of Normal English Males and Females, *J. Hyg.* 33: 36, 1933
11. Osgood, E. E. Normal Hematological Standards, *Arch. Int. Med.* 56: 849, 1935
12. Wintrobe, M. M. Blood of Normal Men and Women. Erythrocyte Count, Hemoglobin and Volume of Packed Red Cells of 229 Individuals, *Bull. Johns Hopkins Hosp.* 53: 118, 1933
13. Osgood, E. E. Hemoglobin Color Index Saturation Index and Volume Index Standards, *Arch. Int. Med.* 37: 685, 1926
14. Foster, P. C., and Johnson, J. R. Oxygen Capacity and Hemoglobin Content of Normal Blood of Men, *Proc. Soc. Exper. Biol. & Med.* 28: 929, 1931.

- 15 Fiddes, J and Witney, C Erythrocyte Count and Hemoglobin Percentage in the Blood of Adult Males in Saskatchewan (Saskatoon), *Canad M A J* 35 (54), 1936
- 16 Price Jones, C The Concentration of Hemoglobin in Normal Human Blood, *J Path & Bact* 34 779, 1931
- 17 Sokhey, S S, Gokhale, S T, Malnadkar, M A, and Billimoria, H S Red Cells, Hemoglobin, Colour Index, Saturation Index, and Volume Index Standards Part I Normal Indian Men A Study Based on the Examination of 121 Men, *Indian J M Research* 25 505, 1937
- 18 Haden, R L Hemoglobin Standards *Am J Clin Path* 3 85 1933
- 19 Orías, O La cantidad de hemoglobina sanguínea en hombres y mujeres adultos sanos en la Republica Argentina, *Rev Soc argent de biol* 6 39, 1930
- 20 Biering, F Nielsen, A, and Nielsen, E Comparative Studies on Haldane's and Van Slyke's Methods for Oxygen Capacity Determination and the Serviceability of These Methods for Standardization of Hemoglobinometers, *Acta med Scandinav Supp* 78 174, 1935
- 21 Walters, O S Normal Erythrocyte Hemoglobin and Packed Cell Volume Standards in Young Men, *J Lab & Clin Med* 19 851, 1934
- 22 Sachs, A, Levine, V F, and Imbann, V A Copper and Iron in Human Blood, *Arch Int Med* 55 227, 1935
- 23 Napier, L F, and Dns Gupta, C R Immunological Studies in Indians Part II Normal Standards for a Bengali Town Population, *Indian J M Research* 23 300, 1935
- 24 Wintrobe, M M Blood of Normal Young Women Residing in a Subtropical Climate, *Arch Int Med* 45 287, 1930
- 25 Osgood, E F and Haskins, H D Relation Between Cell Count Cell Volume and Hemoglobin Content of Venous Blood of Normal Young Women, *Arch Int Med* 39 613, 1927
- 26 Sokhey, S S, Gokhale, S K, Malnadkar, M A, and Billimoria, H S Red Cells, Hemoglobin, Colour Index, Saturation Index, and Volume Index Standards Part II Normal Indian Women A Study Based on the Examination of 101 Women, *Indian J M Research* 25 723, 1938
- 27 Klumpp, T G The Variability of Non Hemoglobin Iron *J Clin Investigation* 14 351, 1935
- 28 Klumpp, T G The Relation of Blood Iron to the Measurement of Hemoglobin Medical papers dedicated to H A Christian Boston, 1936, p 581
- 29 Bing, F C, Hanzal, R F and Myers, V C Hypersideremia Following the Oral Administration of Iron, *J Biol Chem* 109 100, 1935
- 30 Landsay, J W, Rice, F C, and Schinger, M A A Plan for a Standard Method of Estimating and Reporting Hemoglobin Values *J Lab & Clin Med* 11 737 1926
- 31 Kennedy, R P The Quantitative Determination of Iron in Tissues, *J Biol Chem* 74 383, 1927
- 32 Helmer, O M, and Emerson, C P, Jr The Iron Content of the Whole Blood of Normal Individuals, *J Biol Chem* 104 157 1934
- 33 Haden, R L The Determination of Hemoglobin by the Iron Content Method, *J Lab & Clin Med* 19 406, 1934
- 34 Johnson, M, and Hanke, M E The Iron Content and Oxygen Capacity of Blood, *J Biol Chem* 114 157, 1936
- 35 Andes, J E, and Northup, D W Photoelectric Colorimetry I Determination of Blood Iron and Hemoglobin, *J Lab & Clin Med* 24 197, 1938
- 36 Breuer R, and Miltzer, W E A Micromethod for the Determination of Iron in Blood *J Biol Chem* 126 561, 1938
- 37 Morrison, D B, and Hisey, A The Carbon Monoxide Capacity, Iron, and Total Nitrogen of Dog Hemoglobin, *J Biol Chem* 109 233 1935
- 38 Myers, V C Practical Chemical Analysis of Blood, ed 2, St Louis 1934 C V Mosby Co., p 167
- 39 Hanzal, R F Determination of Iron in Biological Material, *Proc Soc Exper Biol & Med* 30 846, 1933
- 40 Bing F C, and Baker, R W The Determination of Hemoglobin in Minute Amounts of Blood by Wu's Method, *J Biol Chem* 92 589, 1931 Bing, F C Purification of Benzidine, and an Improved Reagent, for Estimating Hemoglobin in Blood, *J Biol Chem* 95 387, 1932
- 41 Folin O A New Blood Sugar Method *J Biol Chem* 77 421 1928, Jeghers, H J, and Myers, V C A Reduction in the Amount of Blood Required for the Folin Micro Blood Sugar Method, *J Lab & Clin Med* 15 982, 1930

# COMBINED DIPHTHERIA TOXOID AND TETANUS TOXOID, ALUM PRECIPITATED\*

## PROLONGATION OF ACCELERATED IMMUNITY FOLLOWING STIMULATING DOSE OF ANTIGEN

F. G. JONES AND JAMES M. MOSS  
INDIANAPOLIS, IND.

IN TESTING the value of diphtheria or tetanus toxoid it is the usual custom to test the amount of immunity within a few weeks or months following the injection of the antigen, but very little consideration has been given to the duration of the immunity and to steps that may be taken to prolong the same. Fraser and Brandon<sup>1</sup> found that 14 of 41 children who received three doses of unmodified diphtheria toxoid five years previously were Schick positive, and they recommended that preschool children be given another 1 c.c. of toxoid upon entering school. While this Schick reversion is probably exceptional following three doses of toxoid, it does demonstrate the point in question.

Recently the trend has been to perfect antigens so that only one dose will be required, and because this has been more or less successful in the case of diphtheria toxoid, it has been attempted with many other antigens. It would seem that this is the time to decide how great an immunity is desired and, after that immunity is obtained, how long it should be retained. Dr. Harrison,<sup>2</sup> of the National Institute of Health, after an extensive impartial investigation, recommended the use of alum-precipitated toxoid. Our work<sup>3-5</sup> has indicated that it is the better immunizing agent, but we believe we have now reached the point where the evidence will enable the physician to decide which agent he will use and how many injections he will administer.

The experiments we are now reporting and others to follow are merely to enlighten you further on the advantages and disadvantages of various types of antigen and should not be construed as meaning that there is but one, and one only, that should be used.

One of the first points we would like to present is whether it is advisable to administer all of the antigen in one injection. A report by Benjamin, Fleming, and Ross,<sup>6</sup> together with our investigations, indicates that 1 c.c. of an antigen, when divided into two or three injections, will produce a much higher immunity than when the full volume is given in one injection; furthermore, the immunity will be more prolonged. Fraser and Halpern<sup>7</sup> have shown that if individuals have even a trace of diphtheria antitoxin in their systems, it may be markedly accelerated by making an additional Schick test. The work we are now reporting confirms these observations (Chart 1). Again, this must not be

\*From the Lilly Research Laboratories and Division of Research, Indiana University School of Medicine, Indianapolis.  
Received for publication, June 22, 1938.

taken to mean that one dose of alum-precipitated diphtheria toxoid is not a satisfactory antigen, but it is a suggestion of another method of use Silberschmidt,<sup>8</sup> Leach,<sup>9</sup> Gilmore,<sup>10</sup> Jensen,<sup>11</sup> and others have shown that if some immunity is already present, inhalation of toxoid will increase it. In a recent article Bonsfield and King Brown<sup>12</sup> reported on the use of finely atomized formal toxoid, but the reactions were very severe, and they do not recommend this method.

There is also some evidence that injections of the antigen are usually too close together. A series of closely spaced injections will build up immunity, but if the host is permitted to obtain the full antigenic force of one injection before the second injection is given, an accelerated response is the result. This accelerated response usually produces a higher immunity than a series of injections given at short intervals between doses.

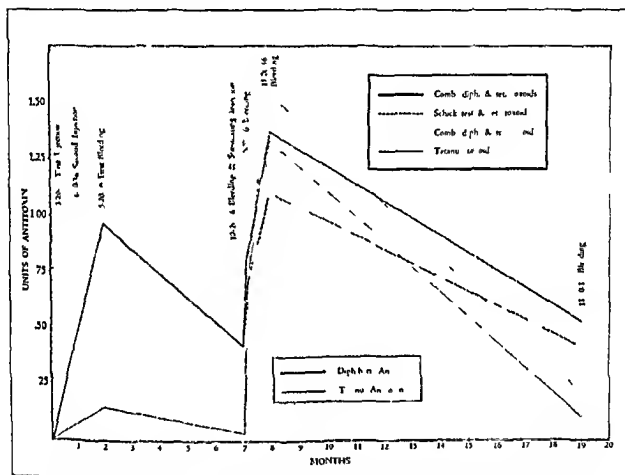


Chart 1—Combined diphtheria toxoid and tetanus toxoid alum precipitated

We recently reported<sup>13</sup> the results obtained from the injection of an alum precipitated combined diphtheria toxoid and tetanus toxoid in a group of medical students. Experiments to date indicate that at least two doses of toxoid are required to produce an immunity to tetanus. These students received two injections (0.5 cc each) one month apart. Bleedings were made at various intervals and the sera were tested for potency.

Six months after the first injection the group of students was divided. Part of them were given the Schick test and then injected with alum precipitated tetanus toxoid. The balance were injected with the combined diphtheria and tetanus toxoids but were not given the Schick test. They were all bled one and four weeks after the stimulating dose, and then sera were tested. Chart 1 shows the marked acceleration of diphtheria antitoxin and tetanus antitoxin. Especial attention is called to the spectacular increase in diphtheria antitoxin produced by the Schick test.

## PROLONGATION OF ACCELERATED IMMUNITY

To ascertain if there was a prolongation of immunity one year after the stimulating dose, these individuals were again bled and their sera tested for diphtheria antitoxin and tetanus antitoxin. The tests for diphtheria antitoxin were made as previously reported<sup>13</sup> by intracutaneous tests on rabbits. Here-  
tofore, the tests for tetanus antitoxin were made with guinea pigs using the standard test, but at this time the tests were made on mice. Toxin and antitoxin were mixed in the usual manner, 1 e.c. of the diluted antitoxin with 2 e.c. of the diluted standard toxin (1 L+ dose). After the mixture stood for at least one-half hour, 0.2 e.c. was injected intravenously into the tail of a white mouse, weighing 15 to 18 gm. The needles used were 27 to 30 gauge. We have found this test to conform quite regularly with the standard guinea pig test.

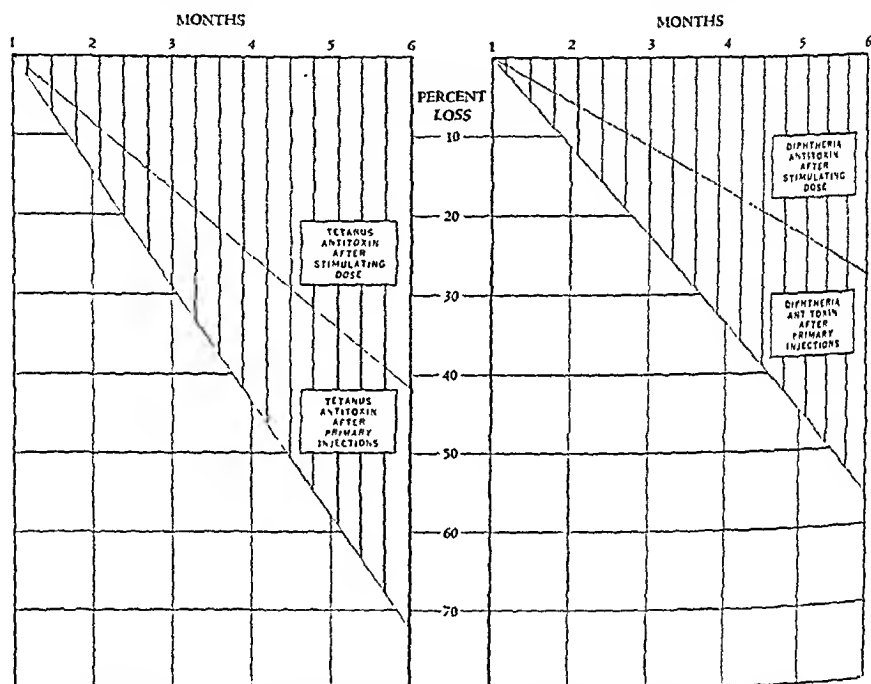


Chart 2.—Combined diphtheria toxoid and tetanus toxoid, alum precipitated.

Chart 1 shows the average antitoxin titer of the group at different periods. There has been a feeling by some that while it is true a marked acceleration follows a stimulating dose, the recession is almost as rapid and the prolongation only apparent because of the increased antitoxic titer. We have, therefore, prepared Chart 2, showing the percentage decrease over various periods of time and definite prolongation of immunity. This chart is based on the potencies for diphtheria and tetanus antitoxin shown in Chart 1. As mentioned before, this group received two injections of the combined diphtheria and tetanus toxoids one month apart. Six months after the first injection the diphtheria antitoxin titer showed a drop of 64 per cent, while six months after the stimulating dose (given six months after the first two injections) the diphtheria

antitoxic titer decreased only 28 per cent. In the same group the antitoxic potency had decreased 72 per cent with tetanus six months after the first two injections, while six months after the stimulating dose the tetanus antitoxic titer decreased only 42 per cent. This titer of antitoxin after one year is so high that there will be certain individuals who probably will not require another injection of tetanus toxoid at the time of mpmv, but such a suggestion should be ignored at this time and every individual should be injected with another dose of tetanus toxoid whenever there is a possibility of a tetanus infection.

The group of students used for this experiment will graduate in medicine this spring so we probably shall not be able to get another series of bleedings next fall. Another group has been started with students who are in their first year of medicine, and it is hoped this group will give us a period of study lasting four years.

The question now is: Will children develop an immunity as high and as prolonged as we have found in adults? In the fall of 1937 a large group of children was injected with the combined diphtheria and tetanus toxoids alum precipitated, and then blood sera will be tested at various intervals. The expectancy is that this group can be followed for a number of years.

#### DISCUSSION

The results of this study would lead one to believe that a stimulating dose of toxoid is of value and that two injections are much better than one even though the total amount injected is not increased. It is indicated that where more than one injection is given, the longer the interval (within certain limits) between injections the higher the immunity that will develop.

In this experiment there was an interval of a month between the injections but we now feel that a two month or even a three month interval would probably have been more effective.

#### CONCLUSIONS

A stimulating dose of alum precipitated combined diphtheria and tetanus toxoids in human subjects has produced not only an immunity that is much greater than that obtained with the two primary injections, but one that is prolonged over a much greater interval.

The use of combined toxoids does not interfere with the specific immunity response.

#### REFERENCES

1. Fraser D. T. and Brandon, K. I. Duration of Schick Immunity. *Canad. Pub. Health J.* 27: 597, 1936.
2. Harrison W. I. Some Observations on Use of Alum Precipitated Diphtheria Toxoid. *Am. J. Pub. Health* 25: 295, 1935.
3. Jones F. G. and Moss I. M. Studies on Tetanus Toxoid. I. The Antitoxic Titer of Human Subjects Following Immunization With Tetanus Toxoid and Tetanus Alum Precipitated Toxoid. *J. Immunol.* 30: 115, 1936.
4. Jones F. G. Duration of Immunity Following Diphtheria Prophylaxis. *J. Lab. & Clin. Med.* 22: 576, 1937.
5. Jones F. G. and Moss I. M. Studies on Tetanus Toxoid. II. The Response of Human Subjects to an Injection of Tetanus Toxoid or Tetanus Alum Precipitated Toxoid One Year After Immunization. *J. Immunol.* 33: 185, 1937.
6. Benjamin, B., Fleming, G., and Ross, M. Results of Schick Test in 5,190 Children Given Injections of Diphtheria Toxoid. *Am. J. Dis. Child.* 55: 51, 1938.

7. Fraser, D. T., and Halpern, K. C.: Serial Titrations of Diphtheria Antitoxin Following Toxoid, *Canad. Pub. Health J.* 26: 476, 1935.
8. Silberschmidt, W.: Immunisierung durch Inhalation, *Schweiz. med. Wchnschr.* 64: 1089, 1934.
9. Leach, C. N.: Field Studies in Diphtheria Immunization, *Chinese M. J.* 49: 771, 1935.
10. Gilmore, C. E.: Diphtheria; Prevention and Control, *Texas State J. Med.* 31: 221, 1935.
11. Jensen, C.: Active Immunization Against Diphtheria by the Combined Subcutaneous and Intranasal Method, *Proc. Roy. Soc. Med.* 30: 71, 1937.
12. Bousfield, G., and King-Brown, W. W.: Diphtheria Immunisation With Finely Atomised Formol Toxoid, *Lancet* 1: 491, 1938.
13. Jones, F. G., and Moss, J. M.: The Antitoxic Titers of Human Subjects Following Immunization With Combined Diphtheria and Tetanus Toxoids, Alum Precipitated, *J. Immunol.* 33: 173, 1937.

# LABORATORY METHODS

---

## A NEW APPARATUS FOR PHOTOELECTRIC RECORDING OF THE COAGULABILITY OF BLOOD AND OTHER PROGRESSING PROCESSES\*

---

K. K. NYGAARD, M.D., Oslo, Norway

---

IN PREVIOUS communications an outline has been given of a test for the determination of the coagulability of the blood plasma<sup>1, 2</sup> and its clinical applicability.<sup>3, 4</sup> Later an improvement of this method was published, using the principle of the photoelectric cell.<sup>5, 6</sup> The modification was based on the observation, that as coagulation takes place, there is an increased density of the specimen under observation. The degree of increased density was registered by a photoelectric arrangement. At first an apparatus was designed, which necessitated the reading of the amperemeter every ten to fifteen seconds (coagelometer). In order to exclude the possibility of errors in the personal visual observations, an apparatus was constructed which graphically recorded the relative change of light during the process of coagulation (Baltes Nygaard coagelograph). This work was originally done at the Mayo Clinic in cooperation with its Physics Department.

By the use of the coagelograph, certain types of tracings were obtained. Certain points of the tracings were found to coincide with important phases of the process of blood coagulation. A first break of the tracing was noted at the time of the first formation of fibrin. A second break was noted at the end of formation of the clot. A last break was apparent at the beginning of retraction of the clot. The tracings, directly representing a graphic expression of the changes of light transmitted through the specimen during its coagulation, were consequently considered indirectly to be an expression of the various phases of the coagulation of the specimen under observation. The tracings were termed coagelgrams.

The original arrangement of the coagelograph required a darkroom in which to operate the apparatus. Because of the sensitivity of the galvanometer used for these investigations, a very stable support for it was essential. For these, and other reasons, the original apparatus lacked certain essential requirements for practical applicability.

The method described has proved of clinical value in studies of changes in the coagulability of the blood where such informations are desired. If, however,

\*From the University Clinic Surgical Department Oslo, Norway. Professor J. Holst chief.

This work was aided by grants from Dr. Alexander Malthes Legation.

Received for publication April 22, 1938.



the procedure was to obtain a more practical applicability, it would be necessary to design an apparatus which satisfied certain practical requirements. Following points were considered of essential importance: Arrangement of the various parts of the apparatus into one transportable unit, requiring minimum of space, to be operated in any laboratory without the necessity of a darkroom, the apparatus still retaining the accuracy and stability of the original arrangement

Efforts in order to overcome these technical difficulties have resulted in the construction of a transportable apparatus which appears to satisfy these requirements. A. Faleh, assistant student, has greatly assisted in the technical perfection of the apparatus. The new type of apparatus is transportable, of the size of a regular electrocardiograph. It is stable and it is ready for use wherever a high frequency alternating current is available.

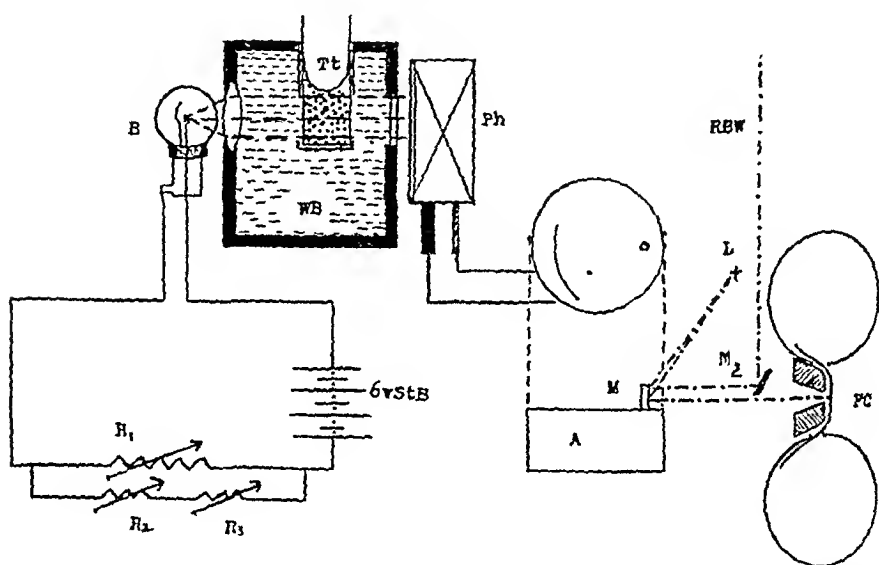


Fig. 1.—Diagram of photograph 6v *STB*, 6 volt lead storage battery;  $R_1$   $R_2$   $R_3$ , adjusting resistances; *B*-35 watt, 6 volt automobile headlight bulb (*Edis*); *WB*, water bath; *Tt*, test tube in position; *Ph*, photoelectric cell, *A*, amperemeter, *M*, mirror of amperemeter reflecting light from bulb; *L*, placed outside darkroom compartment; *M<sub>2</sub>*, individual reflecting mirror, *RBW*, vertically reflected beam of light onto observation window in lid of apparatus; *FC*, film cassette.

#### DESCRIPTION OF APPARATUS

The apparatus may be considered made up of two main parts, the registering and the recording system.

The former deals with registrations of variations of light occurring during the process of coagulation. It consists chiefly of a constant source of light, a water bath, a socket for the test tube with the specimen under observation, and a photoelectric cell.

The latter system deals with a graphic recording of the variations in illumination registered by the photoelectric cell. This part consists of an amperemeter, whose mirror reflects a beam of light onto light-sensitive paper, rotated by a motor at a constant speed per unit time. In order to obtain satisfactory recording, ample consideration has been given to the necessity of obtain-

ing a recording system, built into a darkroom compartment which excludes all stray light. A diagrammatic sketch of the apparatus is given in Fig. 1.

*Source of light.* This consists of a 35 watt automobile headlight bulb (Bilux). It is lighted by a 6 volt lead storage battery, and is placed behind and outside the water bath.

*The water bath.* This is by thermostatic arrangement kept at constant temperature,  $37.5^{\circ}\text{C}$ . for studies of the coagulability of the blood. In the lid of the water bath is a rectangular opening, corresponding to a socket in the water bath, and intended to hold the test tube of identical form. Through a double convex lens in the back, and a window in the front wall of the water bath, parallel light traverses in horizontal direction the bath and the specimen contained in the test tube.

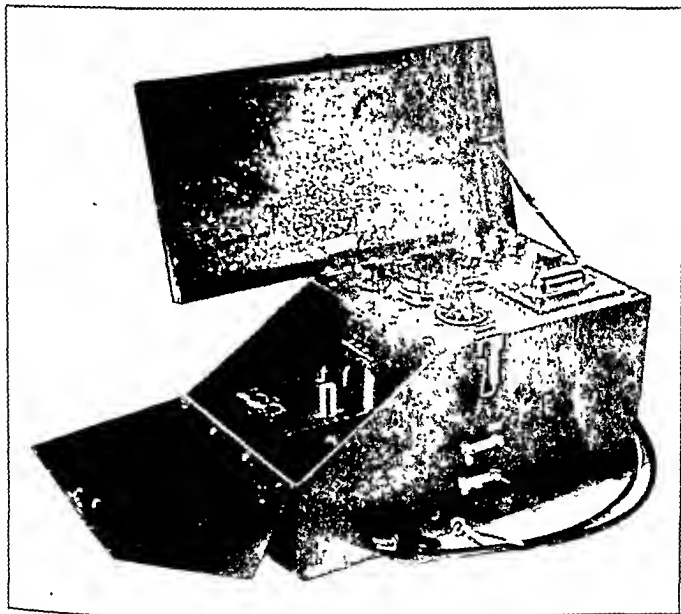


Fig. 2.—Photograph of the apparatus (photomicrograph)

*Test tubes.* These are specially designed with plane-parallel sides. Internal diameter 0.5 and 1.0 cm., and 5.5 cm. high. The test tubes are placed in the water bath with their broadest side exposed to the light.

*Photoelectric cell.* The registering instrument is a photronic cell (Weston model 594). It is placed vertically in front of the water bath.

*Control of illumination.* During the performance of the test a constant illumination is necessary for comparable observations. Adjustment of illumination is obtained by a rheostatic arrangement. The apparatus lends itself readily

to control observations of this constancy of illumination. These control observations are routinely taken before each test is started, as will be dealt with later in this paper.

In order to exclude possible interference from variations of light in the laboratory during the observations, the registering system here dealt with is covered with an individual lid. This lid may be seen opened in Fig. 2.

*The amperemeter.* We have chosen a Weston amperemeter, 100 micro-amperes (model 600). The needle of the galvanometer is replaced by a small mirror which, with its longest axis vertically is placed in the center of the movements of the amperemeter needle. By a special technical arrangement, the whole instrument can be made to rotate horizontally about the axis of the vertical mirror. This arrangement has been chosen in order to assure recording of processes in which an excessive variation of illumination during the observation period otherwise would have caused the recording beam of light to fall outside the photosensitive paper.

*Film.* We are using regular electrocardiographic paper of the perforated type, 6 cm. wide. This paper is made to rotate at the constant speed of 1 cm. per minute by a synchronous motor.

*Darkroom compartment.* This is necessary for a successful recording, and includes mainly the galvanometer and the cassette for the rotating photosensitive paper. From a source of light placed outside this room, a beam of light is admitted through a small opening in its wall onto the reflecting mirror of the amperemeter. This reflected beam of light from the amperemeter is divided in two parts by a reflecting mirror before reaching the recording paper. This particular mirror is placed at such an angle that one part of reflected light from the amperemeter passes directly and horizontally onto the paper. The other part is by the particular mirror reflected in vertical direction onto a specially designed window in the lid of the apparatus. The intention of this arrangement is evident. It permits the observer to follow the deflections of the amperemeter, and to stop the motor when the test is completed.

In order to obtain a recording of time onto the photosensitive paper, the darkroom compartment includes a partly covered source of light which by reflection automatically illumines the exposed part of the paper every ten seconds.

The cassette containing a feeder and a receiver for the photosensitive paper is a removable part of the darkroom compartment. It is built according to a plan similar to that used for the film cassette of the regular electrocardiograph. It is driven by a synchronous motor placed outside the darkroom compartment. After completion of the day's observations, the cassette may be separately removed without danger of exposure, and may be taken to the x-ray department for development and fixation.

## RESULTS

In Fig. 3 is given an illustration of a complete coagelgram, obtained by this new type of apparatus. The result is identical with the previously obtained tracings, with one minor variation. With the new type of coagelgraph, the last phase of blood coagulation, the clot retraction period, is found to follow immediately or shortly upon the completion of the gel-formation period. The rest period, as previously recorded in coagelgrams of the original type, is thereby

considerably shortened. This is a result of the horizontal illumination of the specimen, as compared to the vertical one employed in the original apparatus. The expression of serum begins at the sides of the test tube later reaching the bottom and top layers of the clot, thereby easily accounting for this minor discrepancy.

The technical performance of the test has remained essentially the same.

In one respect this new apparatus affords possibilities for more exact, comparable observations, as compared to the original apparatus. This point may be dealt with a little more fully, as it concerns a most important factor of photoelectric observation.

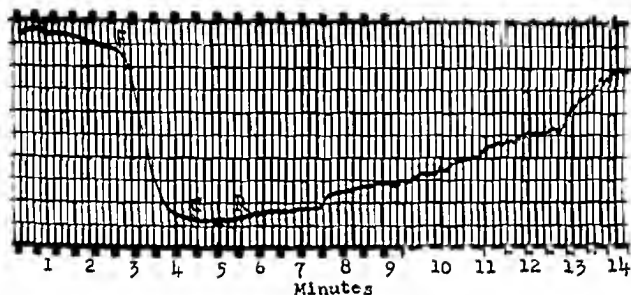


Fig 3—Complete coagulogram of blood plasma. *F* indicates initial formation of fibrin. *C* indicates end of clot formation. *R* indicates beginning of clot retraction. These points of the coagulogram naturally divide the process of coagulation into four phases which have been termed (1) dissociation period (2) gel formation period (3) rest period and (4) clot retraction period.

In general, the current output of the photoelectric cell is considered proportional to the illumination of the photoelectric cell. For exact observations it is imperative that the emission of light is kept constant. In our arrangement, even with the most perfect stabilizer of the source of light, possibilities for variation in illumination of the photoelectric cell still exist. These may be found in inconstant physical properties of the content of the water bath, and of the glass of the test tubes, and, as the main source of error, the color and density of the citrated or oxalated plasma. In order to overcome these possibilities for interference following control, observation is taken before each performance of the test. 0.6 cc of citrated plasma is pipetted off into the test tube, which is then placed in the water bath, without the addition of the calcium chloride solution. By noting the deflection of the mirror of the ampere meter, as evident from observation of the reflected light onto the window in the lid of the apparatus, and further by noting the exact position of the rotated galvanometer, the illumination of the cell may be kept at any constant, arbitrarily chosen value, with the noncoagulated specimen in position by the help of the course and the fine rheostatic arrangement. After this preliminary control observation is performed, one may proceed to the addition of the solution of calcium chloride and the recording of the process.

During the last years, great interest has centered on the applicability of the photoelectric principle to quantitative colorimetry. Sheard and Sandford<sup>7, 8</sup>

several years ago designed a photometer for the quantitative determination of hemoglobin. During the last years Evelyn<sup>9</sup> has worked out a universal photoelectric colorimeter.

It is reasonable to assume that this principle in the near future will be applied to an increasing number of qualitative and quantitative determinations in the medical laboratory. So far, the nature of the methods published has been of the static type, requiring one reading for the actual, final determination. This is in contrast to reactions of the progressing type, an example of which is the process of coagulation of the blood. It is obviously practical to include an automatic recording for the determination of the progress of this latter type of reactions.

The apparatus here described has readily lent itself to observations and recordings of other reactions of progressing nature as well. A report of these investigations is to be given at a later date. In a following paper we are presenting a preliminary report of the application of this apparatus to the reading of the Wassermann reaction.<sup>10</sup>

Having been convinced by our investigations that the apparatus is applicable to a variety of progressing processes, we propose to term this apparatus *photograph*.

#### SUMMARY

A description is given of a new transportable apparatus for automatic photoelectric recording of the coagulability of the blood plasma.

The apparatus is also applicable to other progressing processes, which during their progression effect a variation of light transmitted through the specimen under observation.

#### REFERENCES

1. Nygaard, K. K.: Coagulability of Blood Plasma, Proc. Staff Meet., Mayo Clin. 7: 544, 1932.
2. Nygaard, K. K.: Coagulability of Blood Plasma: A Method of Determining Hemorrhagic Tendency of Jaundiced Patients, Proc. Staff Meet. Mayo Clin. 7: 691, 1932.
3. Nygaard, K. K.: Coagulability of Blood Plasma: Remarks on the Technique of Its Determination, Proc. Staff Meet. Mayo Clin. 9: 150, 1934.
4. Nygaard, K. K.: Coagulability of the Blood Plasma and Changes in the Number of Platelets in Thrombocytopenic Purpura, Proc. Staff Meet. Mayo Clin. 9: 485, 1934.
5. Blades, E. J., and Nygaard, K. K.: Determination of the Coagulability of the Blood Plasma by the Photo-Electric Cell, Proc. Staff Meet. Mayo Clin. 11: 151, 1936.
6. Nygaard, K. K., and Blades, E. J.: Interpretation and Clinical Significance of Coagulograms in Obstructive Jaundice, Proc. Staff Meet. Mayo Clin. 11: 705, 1936.
7. Sanford, A. H., and Sheard, C.: The Determination of Hemoglobin With the Photo-Electrometer, J. LAB. & CLIN. MED. 15: 483, 1930.
8. Sanford, A. H., Sheard, C., and Osterberg, A. E.: The Photometer and Its Use in the Clinical Laboratory, Am. J. Clin. Path. 3: 405-420, 1933.
9. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. 115: 63, 1936.
10. Nygaard, K. K., and Guthe, T.: Automatic Recording of the Wassermann Reaction by the Photo-Electric Principle (Photograph) With Suggestion of a Reading Scale. Aeta med. Scandinav. In Press.

## PREPARATION OF LIVER MEDIA\*

D E HASLEY, MD, AND H SCHMITT  
DETROIT, MICH

THE value of liver media for culturing organisms of the brucella group seems definitely established. They are satisfactory not only for stock cultures, but also for their primary isolation. With the increase in demand for the laboratory to aid in the diagnosis of undulant fever, it is advisable that all laboratories have proper media for the successful cultivation of the brucella organisms.

It has been our experience that many workers encounter difficulty in the preparation of liver media while following directions which have been published up to the present time. It is the purpose of this paper to give a simplified method which may be easily applied, even in those laboratories which have but limited equipment. The method follows the same general technique as is given for the preparation of hormone agar.<sup>1</sup> The ingredients used for liver agar are as follows:

Beef liver	1 pound
Tap water	1000 c c
Peptone	10 gm
Salt	5 gm
Agar	25 gm
Egg, whole	1
N/1 sodium hydroxide	9 c c

The fresh beef liver is passed through a meat grinder and placed in a suitable container. The water, peptone, salt, agar, and egg are added, and with constant stirring the mixture is heated over a free flame to a temperature of 70° C. Fifteen to twenty minutes are usually required to attain this temperature. The sodium hydroxide is then added and the container is placed in an Arnold sterilizer and heated in flowing steam for sixty to ninety minutes. The medium is then removed and the coagulum is carefully loosened from the sides of the container by means of a spatula and allowed to remain at room temperature for five to ten minutes. If the clot is firm and the liquid is poured slowly, a clear fluid will be obtained without the necessity of filtering. It is our routine procedure, however, to pour the medium through a small amount of glass wool loosely placed in a funnel in order to remove the larger particles which may have separated from the coagulum.

The medium is then placed in tubes or flasks as desired and sterilized by autoclaving at 15 pounds pressure for twenty minutes. A slight sediment may appear with autoclaving, but will not interfere with the cultural value of the medium.

\*From the Department of Bacteriology, College of Medicine, Wayne University, Detroit.  
Received for publication April 27, 1938.

## DISCUSSION

The greatest problem encountered in the preparation of liver media has been the fine, floccular pieces of liver tissue which make filtration a difficult process. The method herein described overcomes this difficulty by the formation of a fairly firm coagulum which holds these particles in one large mass. A suitable container appears to be of importance in obtaining this result. We have been using an aluminum water pitcher of 1,800 c.c. capacity with satisfactory results for one liter lots and find it especially suited for pouring the medium



Fig. 1.—*a*, Appearance of coagulum after heating in Arnold sterilizer; *b*, coagulum undisturbed by careful pouring.

slowly without disturbing the coagulated mass, thereby eliminating the necessity of filtering, except for the use of a small amount of glass wool as explained above. Filtering through cotton, paper, etc., is to be avoided.

Twenty-five grams of agar per liter are used to give a firm, dry medium on which the organisms grow best. Better results are accomplished when the granulated form, rather than the shredded form, of agar is used. While bacto peptone has been used in the past with good results, we are at the present time using proteose-peptone.

Huddleson<sup>2</sup> reports that members of the brucella group grow best at or near a pH of 6.6, and it has been our personal experience that media with a pH of 6.6 to 6.8 give an abundant growth, being more suitable than media having a pH of 6.4 to 6.6. We have recommended, therefore, that amount of sodium hydroxide to be added so that the pH of the finished medium will fall in the pH range of 6.6 to 6.8. After many trials in preparing the medium as directed above, we have found that the pH of each mixture before the addition of the sodium hydroxide has been from 6.4 to 6.6, with the addition of 9 c.c. of N/1 sodium hydroxide per liter the pH rises to 6.8 to 7.0, and with autoclaving the pH falls to the required 6.6 to 6.8 range. We believe that these same pH values will be found in other laboratories, however, we advise that you check the values at the stages mentioned and determine the exact amount of sodium hydroxide necessary to obtain a final pH of 6.6 to 6.8 under your particular working conditions.

The same directions are followed for the preparation of liver broth, the ingredients are the same except that no agar is used. We find that 9 c.c. of N/1 sodium hydroxide per liter gives a final pH in the 6.6 to 6.8 range.

#### SUMMARY

A simplified method for the preparation of liver agar and liver broth has been described.

#### REFERENCES

- 1 Hinton, F. M. *Hormone Media* J Infect Dis 23 169, 1916
- 2 Huddleson, I. F. *Procedures for the Detection of Brucella in Milk* Suppl Am J Pub Health 26 118, 1936

## AN APPARATUS FOR CONTINUOUS INTRAVENOUS INJECTIONS IN UNANESTHETIZED ANIMALS\*

ALFRED STENDEL, JR., M.D.,† AND HARRY M. VARS, PH.D.  
PHILADELPHIA, PA.

IN THE investigation of methods for the control of hypoproteinemia it became necessary to utilize an apparatus which would permit of continuous intravenous injections. Since the injected solutions were given over a period of days, it was essential that the animals be allowed as much freedom of movement as possible. We were particularly interested in using a method which permitted various rates of flow, freedom of movement of the animal, sterility, and simplicity. The apparatus suggested by Jacobs<sup>1</sup> was first constructed, but it proved unsuitable for continuous use, and not flexible enough for specific rates of flow. We, therefore, devised in part, a new apparatus, utilizing such parts of the Jacobs<sup>1</sup> set up as proved efficient in our hands.

\*From the Harrison Department of Surgical Research School of Medicine University of Pennsylvania.

†Lisman Fellow in Medicine

Received for publication May 13 1935



A large Erlenmeyer flask (two liter size) (Fig. 1, *A*), acting as a reservoir for the injection fluid, was connected to the intake of the pump (Fig. 1, *B*) by means of a glass tube extending from the bottom of the flask up through a two-hole rubber stopper. The air passing into the flask was kept bacteria-free by filtering through cotton.

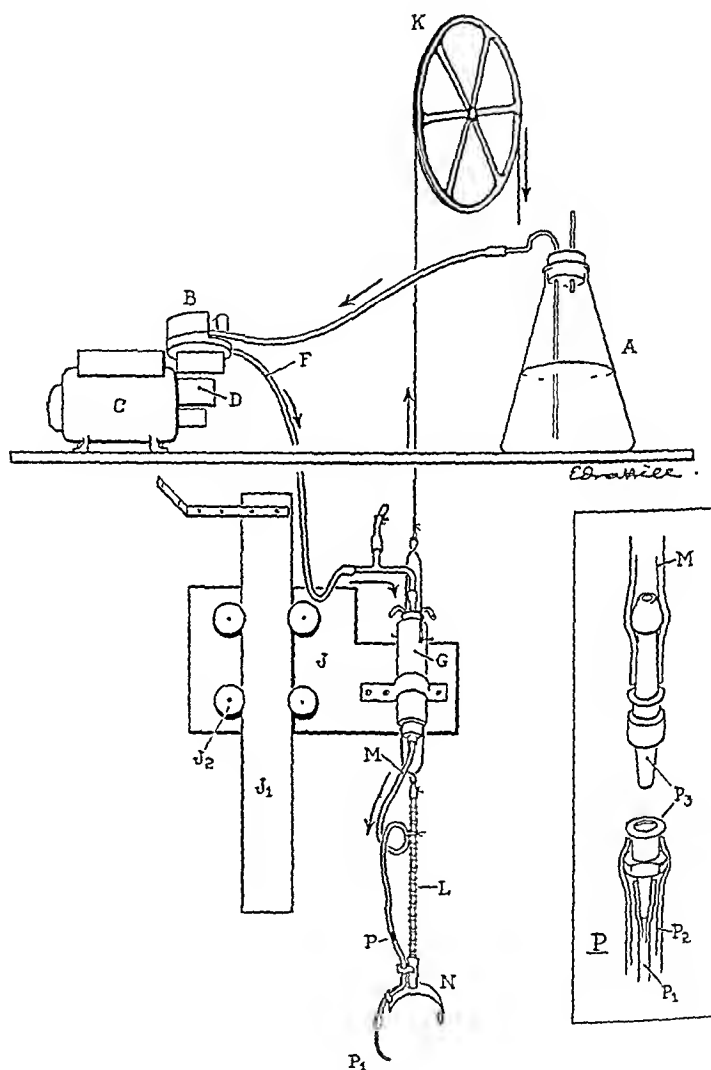


FIG. 1.

We incorporated in the mechanical pump (Fig. 1, *B*, *C*, and *D*; Fig. 2, *B*) the principle utilized by DeBakey<sup>2</sup> in his transfusion apparatus. An electric motor, driven by direct current (Fig. 1, *C*), equipped with a speed reducer (Fig. 1, *D*), ratio 3,300:1, furnished the power used to drive a vertical shaft (Fig. 2, *B* 1) on which were mounted two vertical metal rollers (Fig. 2, *B* 2, 3). These rollers revolved about the central shaft in such manner that one was at all times compressing the specially constructed DeBakey rubber "pumping" tube (Fig. 2, *E*) against a series of metal collars (Fig. 2, *B* 4) which clamped a

flange attached to the tubing (Fig 3, *E 1*) Three bolts (Fig 2, *B 5*) passing through each collar held the flange (Fig 2, *E 2*), serving thus to hold the pumping tubes firmly in place To facilitate the dismantling of the pump and the removal of the pumping tubes when not actually in use, the mounting of one of the metal rollers (Fig 2, *B 6*) was arranged so that it could be pivoted (in toto) about the central shaft

The motor speed was controlled by a suitable rheostat and a revolution counter was attached to the shaft bearing the rollers The amount (volume) pumped per revolution per tube was 23 cc Three pumping tubes could be in simultaneous use for separate injections the fluid being forced by the rollers through the tubes in a clockwise direction

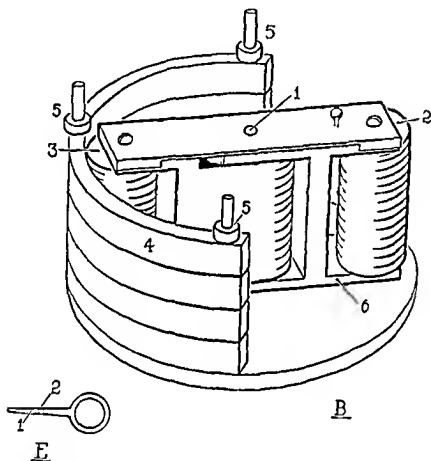


Fig 2

The output of the pump was connected by rubber tubing (Fig 1, *I'*) to the top of a Jacobs' swivel unit (Fig 1, *G*) A glass "T" tube and clamp (Fig 3, *G 8*) served to trap air bubbles The swivel (Fig 3, *G* and *H*) consisted of a small glass syringe (Fig 3, *H 1*) mounted on the top of a hollow metal tube (Fig 3, *H 3*) The top and bottom of the plunger were cut off and the parts well lubricated The metal tube turned freely in an outer metal housing (Fig 3, *H 4*) and the syringe was kept in position by a cap (Fig 3, *G 5* and *H 5*) secured by springs (Fig 3, *G 6*) attached to the body of the outer housing With minor modifications it was a duplicate of that described by Jacobs<sup>1</sup> The metal housing was, in turn, mounted on a carrier arm (Fig 1, *J*) swung from the side of the cage in such a way that by means of a vertical metal track (*J 1*) and roller bearing wheels (Fig 1, *J 2*) mounted on the arm, upward and downward movements alone were possible The carrier arm was counterweighted by passing a strong wire from the top of the swivel (Fig 3 *G 7*) over a large wheel (Fig 1 *K*) mounted above the cage

A sprocket chain (Fig. 1, *L*) and rubber tubing (Figs. 1 and 3, *M*) served to connect the swivel to the dog. The function of the chain was to make certain that as the dog turned about, the swivel would also turn. This was effected by fastening the lower end of the chain to a bolt (Fig. 4, *N* 1) on a curved brass plate (Fig. 4, *N* 2) incorporated in a plaster collar about the dog's neck.

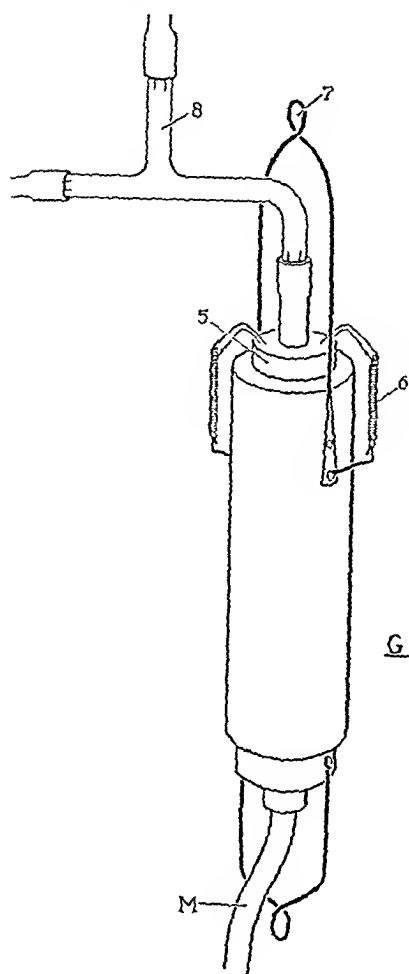
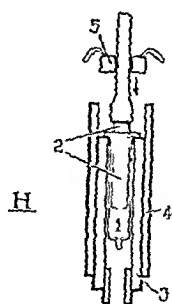


FIG. 3.



H

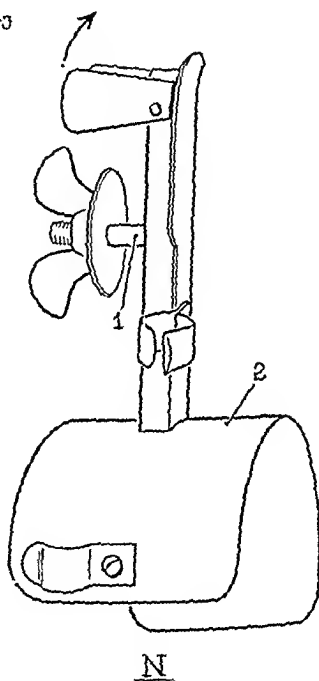


Fig. 4.

The actual injection takes place through a soft rubber catheter (Fig. 1, *P* 1) fixed in the external jugular vein. The catheter and its protecting rubber sheath (Fig. 1, *P* 2) were run from the side of the neck, along the metal plate, through the plaster collar, and finally up along the chain. The catheter was attached by a suitable adaptor (Fig. 1, *P* 3) to the ascending rubber tube (Figs. 1 and 3, *M*), the junction being held firmly by a small tube clamp.

The fluid is pumped from the flask to the external jugular vein, through the "pumping" tube, the swivel, the lower tubing, and the rubber catheter. At the start of each experiment due precautions were taken to insure the absence of air bubbles from any part of the route of fluid flow and assemblage was effected under sterile precautions.

This apparatus has been repeatedly used for continuous intravenous injections in dogs, one for a period of fourteen days. They were able to wander about their metabolism cages, lie down, stand up, and turn about, without any difficulty. Urine was collected throughout the experiments, and the dogs were etherized at suitable intervals without difficulty. Blood samples were taken duly without inconvenience.

Injections were made at rates varying from about 25 cc to as much as 60 cc or more per hour. A number of solutions have already been tried. All the parts were easily sterilized, and sterility was maintained for the duration of the injections.

#### REFERENCES

- 1 Jacobs, H. R. D. Apparatus for Constant Intravenous Injection Into Unrestrained Animals, *J. Lab. & Clin. Med.* 16: 901, 1921.
- 2 DeBaey, M. E. Simple Continuous Flow Blood Transfusion Instrument. New Orleans M. & S. J. 87: 286, 1924.

### PHOTOELECTRIC COLORIMETRY\*

#### III QUANTITATIVE DETERMINATION OF BLOOD GLUCOSE, BLOOD CHOLESTEROL, SERUM PHOSPHORUS, PLASMA PROTEINS AND URINE SUGAR

JEROME E. ANDER, PH.D., M.D., and DAVID NORTHUP, PH.D.  
MORGANTOWN, W. VA.

A DISCUSSION and description of photoelectric colorimeters have been given in the two preceding papers of this series.<sup>1</sup> We only wish to state that the methods listed below are not new methods but are standard procedures adapted for use with a photoelectric colorimeter. In addition, we have given curves for use in each procedure and pointed out the type of filter that we have found most satisfactory.

#### USE OF THE INSTRUMENT

Details for using the colorimeter are given in considerable detail in both preceding papers,<sup>1</sup> but for convenience will be briefly repeated. The instrument should always be turned on fifteen to twenty minutes before it is used to allow it to "heat up," and thereby reach a constant output. The glass cup or cell is then filled with water (or a blank of the reagents), placed in the instrument, the milliamperage output of the photoelectric cell set at some definite value (0.40 Ma.), and the galvanometer adjusted to the neutral point with the transmission set at 100 per cent. Then the cup is filled with the solution to be tested and the light transmission observed, the output of the photoelectric cell being held at 0.40 Ma. all the time. From the curve plotted for this determination, the concentration of the unknown is read off directly.

\*From the Departments of Pathology and Physiology, West Virginia University Medical School, Morgantown.

Received for publication May 19, 1938.

<sup>1</sup>A picture and a complete description of the instrument are given in the first page of this series.

If filters are used, the current output of the photoelectric cell is too low to be read accurately; consequently, the output is adjusted (0.40 Ma. or some convenient figure) without the filter being in place and the voltage noted. Then by means of a voltage regulator, the incoming voltage is held at this value all through the adjustment of the instrument (with the filter in place) and the reading of the unknown. Of course, if the voltage is relatively constant, this voltage regulator is not needed. Additional points in the use of the instrument are given in the procedures listed in this report.

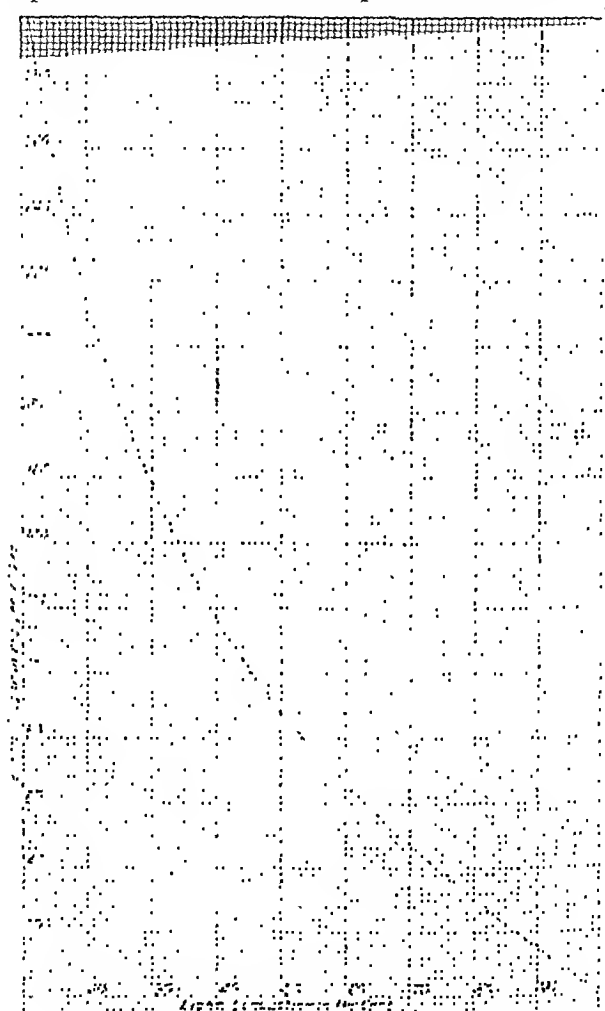


Fig. 1.—Photoelectric emission curve for the blood glucose determination (no filter).

#### BLOOD GLUCOSE

**Principle.**—Blood proteins are precipitated by whatever procedure is desired.\* The 1:10 blood filtrate is boiled with Folin's alkaline copper reagent<sup>†</sup> in a Folin-Wu sugar tube. Cuprous oxide is formed in proportion to the amount of reducing substances (sugars) present, which when treated with phospho-

\*The usual precipitating agent used is tungstic acid (seven volumes of water, one volume of blood, one volume of 10 per cent sodium tungstate, and one volume of  $2/3$  N sulfuric acid). However, tungstic acid filtrates give values about 20 mg. too high. If zinc or cadmium is used as a precipitating agent, the nonglucose reducing substances are removed along with the protein, and the final values are very close to the actual glucose concentration.

molibdic acid forms a blue color in the solution. From the degree of light conduction of the blue solution, the sugar concentration is obtained from the curve.

*Procedure*—Pipette 2 cc of 1:10 protein free blood filtrate into a Folin Wu sugar tube, and add 2 cc of alkaline copper solution\*. Heat in a boiling water bath six minutes, place immediately in cold water for about three minutes, and add 2 cc of phosphomolybdic acid†. When the bubbling has entirely ceased (one to two minutes), make up to volume, mix well and find the light transmission of the blue solution without a filter.

In making the photoelectric measurements adjust the instrument at 100 with a current of 0.40 Ma without using a filter, and with the cell (cup) filled with distilled water. Using the value for the light transmission of the unknown solution, the glucose concentration‡ is obtained directly from the curve in Fig. 1.

#### SUGAR IN URINE

*Principle*—Urine that has been shown to contain sugar by the qualitative Benedict test may be analyzed for glucose in the same manner as for blood. If protein is present, it must first be removed.

*Procedure*—After the urine has shown a qualitative test for glucose, test for the presence of protein. If protein (more than a trace) is present transfer 1 cc of urine to a 50 cc volumetric flask, and add 20 to 30 cc of water followed by 1 cc of 10 per cent sodium tungstate and 1 cc of 2/3 N sulfamic acid. Make up to volume, mix, and filter. If protein is absent, dilute 1 cc of filtered urine to 50 cc with distilled water.

Using 2 cc of the 1:50 diluted urine proceed as with blood filtrate. The value obtained from the curve is multiplied by 5 to give the glucose concentration in mg per 100 cc of urine. If a very high sugar content is known to be present (from the qualitative test), dilute the urine to 1:100 instead of 1:50, and multiply the final value by 10.

#### BLOOD CHOLESTEROL§

*Principle*—The blood or serum is dried on a suitable medium, the cholesterol extracted with chloroform, and the color developed by means of the Liebermann-Burchard reaction. From the degree of light transmission of the colored solution, the cholesterol concentration is obtained directly.

*Procedure*—Accurately measure 0.5 cc of blood (or serum) into an evaporating dish and thoroughly mix with about a teaspoonful of fresh plaster of Paris. Allow to stand about thirty minutes (or heat fifteen minutes at 120°C)¶. Now transfer to an extraction thimble of a suitable size to fit into the ex-

\*Alkaline Copper Solution. Dissolve 40 gm of anhydrous sodium carbonate in about 400 cc of water and add 7.5 gm of tartaric acid. When the latter has dissolved add 40 gm of copper sulfate crystals and mix. Transfer to a 1000 cc volumetric flask and make up to volume. If a precipitate forms on standing (due to impurities) decant into another bottle.

†Phosphomolybdic Acid. To 35 gm of molibdic acid and 5 gm of sodium tungstate in a 1 liter beaker add 200 cc of 10 per cent sodium hydroxide and about 200 cc of water. Boil vigorously for thirty minutes or longer (to remove any ammonia present in the acid), cool, dilute to about 350 cc and add 125 cc of 85 per cent phosphoric acid. Dilute volume to 500 cc.

‡Values above 300 mg cannot be determined if a higher glucose content is present in the sample. Dilute with water before making the determination. The dilution with water gives the true value.

§This is essentially the method of Myers and Wardell modified to fit the photoelectric colorimeter. It is similar to the author's previous method\*. See also the method of Bernhard and Dreker†.

¶Bernhard and Dreker† found that the blood or serum may be dried on filter paper but if such is done the extracting medium must be a mixture of absolute alcohol and ether. The solvent is evaporated to dryness after extraction is completed.

¶his method (directly) if with one or more volumes from the curve multiplied

traction apparatus shown in Fig. 2,\* and insert in the extraction tube. Add about 10 c.c. of cholesterol (or alcohol-ether mixture†) and extract for thirty minutes. Remove the condenser tube, lift the extraction tube a few centimeters above the surface of the hot plate, and allow the tube to evaporate to dryness to remove traces of water. Now add chloroform to the 20 c.c. mark, and then add 4 c.c. of acetic anhydride and 0.4 c.c. of concentrated sulfuric acid. Mix and find the minimum value for the light conduction in the photoelectric colorimeter (lowest reading). The greatest intensity of color is reached between fifteen and twenty minutes from the time the reagents are added.

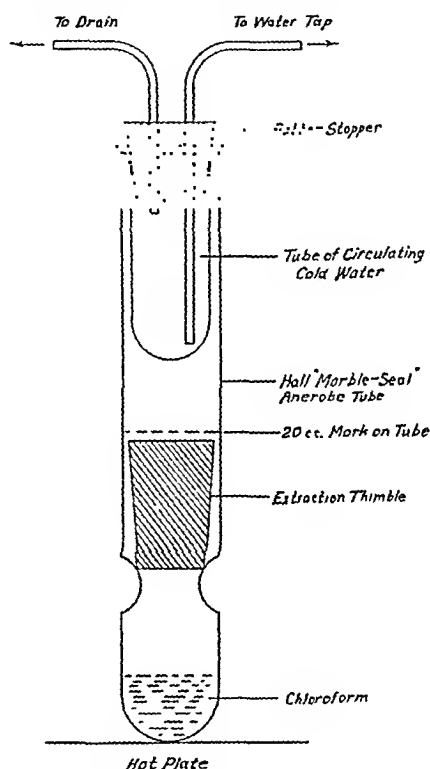


Fig. 2.—Cholesterol extraction apparatus.

The colorimeter is previously adjusted to 100 with a No. 29 Wratten filter (using 0.40 Ma. current without the filter) with water in the cup. From the minimum light transmission value, the concentration of cholesterol is obtained directly from the curve in Fig. 3.

#### SERUM PHOSPHORUS‡

*Principle.*—The protein-free serum filtrate is treated with molybdic acid and a reducing agent, and the light transmission of the resulting blue solution measured.

\*These thimbles may be conveniently made of filter paper and library paste. The extraction apparatus is made from a Hall "marble seal" anaerobe tube (or a similarly shaped tube of a similar size) by marking the 20 c.c. level with a file and assembling the other parts as shown.

†See footnote on page 531.

‡This is essentially the method of Youngburg and Youngburg\* modified to fit the photoelectric instrument.

*Procedure*—To 1 c.c. of blood serum, add 5 c.c. of distilled water and 4 c.c. of 20 per cent trichloroacetic acid. Mix well and filter through ashless filter paper (Whatman No. 40), refiltering if necessary to get a clear solution. Transfer 5 c.c. of the filtrate to a 25 c.c. volumetric flask, and add 4 c.c. of molybdate reagent\* and 1 c.c. of diluted stannous chloride solution†. Mix, let stand fifteen

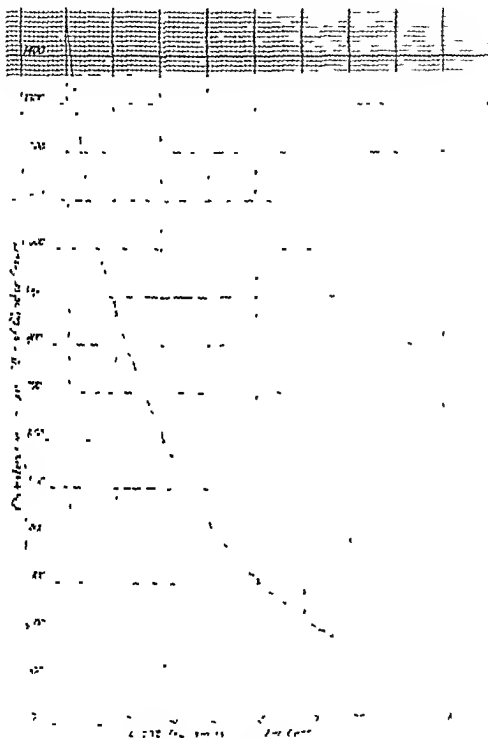


Fig. 3—Photoelectric light transmission curve for blood cholesterol determination (No. 63 Wratten filter)

minutes, and dilute to volume (25 c.c.). Determine the light transmission as soon as conveniently possible. The colorimeter is equilibrated to 100 *without* a filter (using 0.40 Ma) with water only in the cup. The concentration of

\**Molybdate Reagent* Prepare by mixing one volume of 10 N sulfuric acid one volume of 7.5 per cent sodium molybdate and two volumes of water.  
 †*Stannous Chloride Solution* Prepare a stock solution by dissolving 10 gm. of stannous chloride in 25 c.c. of concentrated hydrochloric acid. Keep in a brown bottle (in a refrigerator if convenient). The diluted solution is prepared by diluting 1 c.c. of the stock solution to 100 c.c. with water. The latter must be prepared fresh every day.



serum phosphorus is obtained directly from the curve in Fig. 4. For values above 10 mg., dilute the blood filtrate with an equal volume of water and re-determine.

#### SERUM OR PLASMA PROTEINS\*

*Principle.*—The total proteins and albumin are determined by digestion with selenium digestion mixture and estimation of the nitrogen by nesslerization. The globulin is determined by the difference. The albumin and globulin fractions are separated by 22 per cent sodium sulfate.

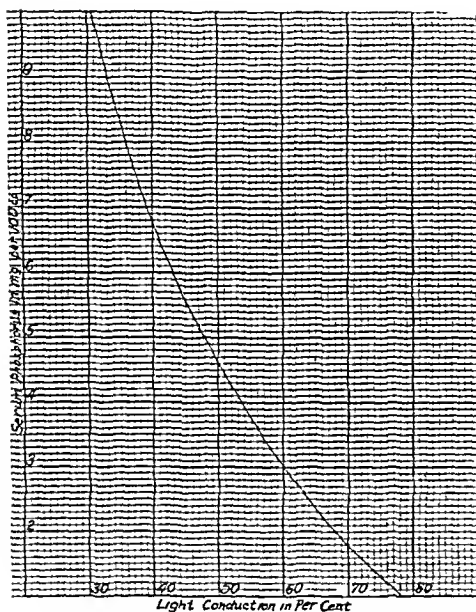


Fig. 4.

Fig. 4.—Photoelectric light transmission curve for serum phosphorus determination (no filter).

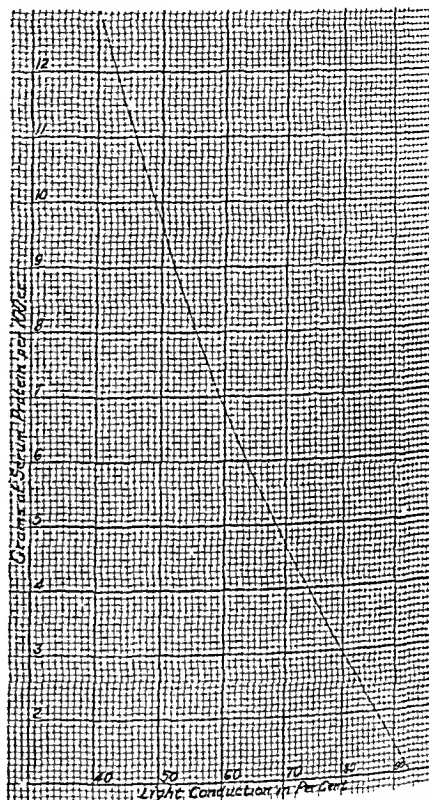


Fig. 5.

Fig. 5.—Photoelectric light transmission curve for estimating plasma proteins (No. 47 Wratten filter).

*Procedure.*—(a) *Total Proteins.* Dilute 1 c.c. of plasma or serum with water (or 0.85 per cent sodium chloride) to 100 c.c. To 1 c.c. of this material in a pyrex tube (graduated at 50 c.c.—a nonprotein nitrogen tube will serve admirably), add 1 c.c. of the selenium digestion mixture† and digest over a small flame under a hood. When the dense white fumes begin to fill the tube reduce the flame to a minimum, preferably not letting the flame actually touch the digestion tube. Do not cover the tube with a watch glass or funnel.‡ Continue

\*A combination of the points in a number of methods with a few additions. See Howe,<sup>9</sup> Wu,<sup>10</sup> and Reis and Powers.<sup>11</sup>

†Selenium Digestion Mixture. Mix 75 c.c. of concentrated sulfuric acid with 25 c.c. of 85 per cent phosphoric acid and add the mixture to 100 c.c. of water. Cool somewhat and add 100 mg. of selenious acid. The solution should be colorless.

‡Covering the flask prolongs the time of digestion by allowing moisture to condense and fall back into the tube. With care and rapid digestion there should be practically no precipitate of dissolved glass when digestion is completed.

the digestion until the material is essentially clear. Occasionally a slight brown flocculation (selenium?) remains in the upper part of the tube. Neglect this as it floats on the surface of the liquid all through the succeeding steps. The entire digestion period should occupy about four to five minutes.

Cool and dilute with water to about 30 cc, add 15 cc of Nessler's solution,\* and make up to volume (50 cc) with water. Mix at once and determine the light transmission using a No. 47 Wratten light filter and a current of 0.40 V<sub>1</sub> (without the filter in place). The colorimeter is first adjusted at 100 with the cup filled with a blank of the reagents (1 cc of digestion mixture + 34 cc of water + 15 cc of Nessler's solution).

The concentration of protein is found directly from the curve in Fig. 5. From this value must be subtracted a correction for the protein equivalent of the nonprotein nitrogen. For bloods with an approximately normal nonprotein nitrogen concentration, this correction is approximately 0.20 gm of protein. If the nonprotein nitrogen is greatly elevated its concentration should be actually determined in the plasma (or serum), using the method given in paper II,<sup>1</sup> and multiplying the value so obtained by 0.00625 to give the protein correction.

**Procedure for Albumin.**—Add 1 cc of plasma (or serum) to 60 cc of 22 per cent sodium sulfate,<sup>†</sup> mix and allow to stand one to two hours or until the protein begins to flocculate. It is best kept in an incubator (37° C) during this time. Filter through No. 40 Whatman filter paper (or some very retentive paper). The first part of the material should be refiltered several times if the filtrate is cloudy and the first 30 cc or more should be discarded.<sup>‡</sup>

One cubic centimeter of the filtrate is digested and nesslerized as with total protein. The value from Fig. 5 is multiplied by 0.61 and the correction for the nonprotein nitrogen (0.20 gm for normal bloods) subtracted as for total protein. This gives the albumin concentration.

The globulin concentration is found by subtracting the albumin from the total protein, the value being for serum or plasma globulin depending on which substance was analyzed.

## DISCUSSION

The desirable features of photoelectric colorimetry have been adequately discussed in papers I and II of this series. The increased accuracy and rapidity, the freedom from eye-strain, and the elimination of the use of simultaneous standard solutions are very notable points. However, a few additional comments regarding the determinations listed in this paper are indicated.

It must be pointed out that the range of sugar values from 0 to 300 mg is entirely covered. Only higher values require a second analysis. In this copper-sugar reaction there is a notable deviation from the actual value when the con-

\*Nessler's Solution. Prepare the double iodide according to the directions given in paper II of this series.<sup>1</sup> Such directions are also given in most laboratory textbooks and manuals. The double iodide keeps indefinitely in brown bottles. The working solution is prepared by mixing one volume of the iodide with one volume of water and adding four volumes of 10 per cent sodium hydroxide. This solution should be prepared fresh every two to three months. It is the same as the solution used in the nonprotein nitrogen determination in paper II.

<sup>†</sup>This solution should be kept at a temperature of about 3° C to prevent precipitation. <sup>‡</sup>Albumin adheres to the filter paper. After about 20 to 30 cc have been filtered the paper becomes saturated with the albumin and the remainder of the filtrate passes through unchanged.

centration of the standard solution (as in the regular Folin-Wu procedure) is not moderately close to that of the unknown; hence to *actually* cover this entire range requires at least four or more standards instead of the two commonly used. The copper reagent deteriorates somewhat with age and should, therefore, be made up fresh about every six months; however, even using a reagent eighteen months old we have found the values obtained as close (on the average) to the actual figure as may be expected with the regular procedure.

In the cholesterol method outlined, the range from 0 to 1,500 mg. per cent is adequately covered. In the regular colorimetric methods, a number of simultaneous standard solutions would be required to cover this range. A more important point is that in the calibration of the curve (and in the analysis of unknown solutions) the lowest transmission figure (most green color) is used instead of the color present at a specified time. We believe this to be an important point as the time of maximum color production varies with a number of factors, making an inherent error in comparative methods.

The use of three simultaneous standards is advised in the standard serum protein procedure; an even greater number would be necessary if high values are to be determined. Also, the time required to prepare three standards is about equal to that in making up the unknown. Yet another point is the deterioration of phosphorus standard solutions, even if a preservative is added. The photoelectric procedure eliminates all these objectionable points.

The selenium digestion mixture works excellently and obviates the necessity of the use of hydrogen peroxide (and its inherent errors). Digestion is complete and a colorless solution is produced.

As a final point we wish to state again that for greatest accuracy (and always whenever feasible) the laboratory worker should construct his own curves by plotting the results with standard solutions. However, we believe that the curves listed can be employed (using similar instruments and equipment) where values within 5 per cent ( $\pm 5$  per cent) are satisfactory. It is doubtful if even this variation will be found.

#### SUMMARY

1. Photoelectric methods are described and conduction curves given for the quantitative determination of blood sugar, cholesterol, phosphorus, and plasma proteins, and for sugar in the urine. The methods described are standard procedures modified to fit photoelectric colorimetry.

2. The advantageous points of these procedures are briefly discussed.

#### REFERENCES

- Andes, J. E., and Northup, David: Photoelectric Colorimetry. I. Determination of Blood Iron and Hemoglobin, *J. LAB. & CLIN. MED.* 24: 197, 1938.  
Northup, David, Hawk, Fabry L., and Andes, Jerome E.: II. Quantitative Determination of Blood Nonprotein Nitrogen, Urea Creatinine, Creatine, Guanidine, and Amino Acids, *J. LAB. & CLIN. MED.* 24: 432, 1939.
- Folin, O., and Wu, H.: A Simplified and Improved Method for Determination of Blood Sugar, *J. Biol. Chem.* 41: 367, 1920.
- Somogyi, M.: Notes on Sugar Determination, *J. Biol. Chem.* 70: 599, 1926.  
A Method for the Preparation of Filtrates for the Determination of Sugar, *J. Biol. Chem.* 86: 655, 1930.
- Fujita, A., and Iwatake, D.: Blood Sugar After Deproteinization With Cadmium Hydroxide, *Biochem. Ztschr.* 242: 43, 1931.

- 5 Myers, V. C., and Wardell, E. L. Colorimetric Estimation of Cholesterol in Blood With a Note on the Estimation of Coprosterol in Feces, *J Biol Chem* 36 147, 1918
- 6 Andes, J. F. A Modified Method for the Determination of Blood Cholesterol, *J LAB & CLIN MED* 20 410, 1935
- 7 Bernhard, A., and Drekter, J. T. A Simple and Accurate Method for the Determination of Cholesterol in Blood Serum or Plasma, *J LAB & CLIN MED* 16 1225, 1931.
- 8 Youngburg, G. E., and Youngburg, M. V. Phosphorus Metabolism I A System of Blood Phosphorus Analysis, *J LAB & CLIN MED* 16 158, 1930
- 9 Howe, P. E. The Determination of Proteins in Blood A Micro Method, *J Biol Chem* 49 109, 1921
- 10 Wu, H. A New Colorimetric Method for the Determination of Plasma Proteins, *J Biol Chem* 51 73, 1922
- 11 Reis, F., and Powers, H. H. Selenium as a Catalyst in the Determination of Non Protein Nitrogen in Blood, *J LAB & CLIN MED* 11 1204 1935

## A CLINICAL GAUGE FOR SENSITIVITY TO PAIN\*

EDWARD HOLLANDER, M.D., NEW YORK, N. Y.

**S**ENSITIVITY to pain varies considerably in different individuals. As pointed out by Libman,<sup>1</sup> it is important to know how a patient reacts to painful stimuli in order to properly evaluate the symptoms presented, especially in gastrointestinal and cardiac diseases. It is also helpful in planning many forms of treatment to know how a patient reacts to pain. Algometers such as are used in neurologic investigations, are not practicable for routine clinical examinations. Libman tests the sensitivity to pain by pressing the finger on the styloid process beside the mastoid bone. However the degree of pressure that the finger exerts is not determined and is therefore an uncontrolled variable in the test.

The appliance illustrated is employed in a simple manner with a blood pressure machine to determine the level at which a patient reacts to a painful stimulus. It consists of an elliptical metal grater, 3 inches by 4 inches in size which fits into a blood pressure cuff. It is held in situ by two slits in adhesive tape (1), into which two flanges of the grater (2) are inserted. The adhesive tape is attached to the outside and the inside of the blood pressure cuff (3). The cuff is inflated slowly, at the rate of about 10 mm. of mercury pressure per second. As the pressure is increased, the prongs of the grater indent but do not puncture the skin, even when the pressure is raised to the limit of the machine (260 mm. mercury). The individual is not informed what is being done so that a spontaneous, unconditioned reaction to the examination is obtained. After the test the metal grater is easily removed from the cuff, which can then be used for measuring blood pressure.

The pressure on the grater prongs that causes the patient to wince the eye involuntarily is recorded as the sensitivity level to pain. Some patients make a verbal protest before wincing, such as "that hurts" or "that sticks" and the pressure reading at such a moment is also taken as the sensitivity level to pain.

\*From the Manhattan State Hospital, New York.  
Received for publication May 24 1938

In hyposensitive persons, no wincing nor objection to the test occurs, even though the limit of the mercury column is reached (260 mm.).

One hundred patients whose reactions to pain were known, and who could be classified as hypersensitive, hyposensitive, or normally sensitive, reacted to this test as follows:

Hypersensitive group (27 per cent), the sensitivity level was below 110 mm. pressure.

Hyposensitive group (29 per cent) showed no sensitivity to pain even at 260 mm. pressure.

Normally sensitive group (44 per cent), the sensitivity level was between 110 and 260 mm. pressure.

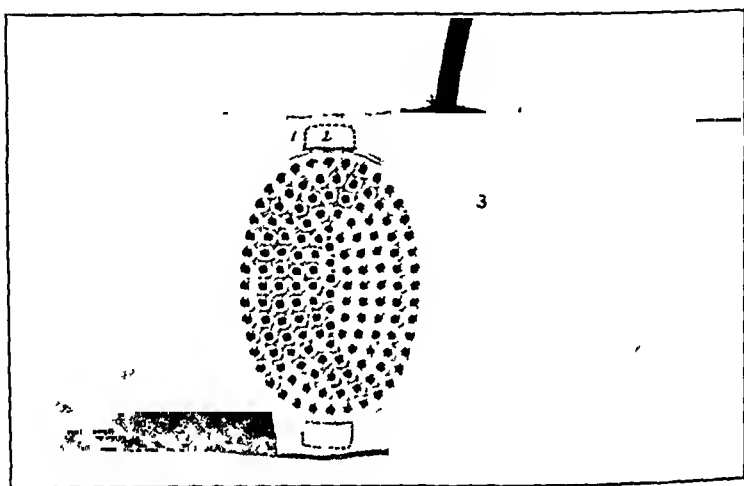


Fig. 1.

In the hypersensitive group, extremely sensitive patients reacted as low as 40 mm., though the majority in this group reacted about 90 mm. In the normally sensitive group, the majority of patients reacted about 150 mm.

This gauge was made by the W. A. Baum Co., New York.

#### REFERENCE

1. Libman, E.: Observations on Individual Sensitiveness to Pain, J. A. M. A. 102: 335, 1934.

THE ROUTINE USE OF CONCENTRATION AND CULTURE METHODS  
FOR THE DETECTION OF TUBERCLE BACILLI IN MICRO-  
SCOPICALLY NEGATIVE SPUTA AND OTHER  
BODY FLUIDS\*

ELIZABETH PETRAN, PH D, AND C ALFRED PERRY, SC D  
BALTIMORE, MD

DURING the past few years a study has been made on the use of concentrative and cultural methods for the recovery of acid fast bacilli from sputa and such body fluids as pleural, spinal, urine, pus etc found to be negative on direct microscopic examination. While the number of extra positives obtained with sputa after concentration or culture has not been large it is felt that the additional time and materials required have been justified. The methods used and the results obtained during 1937 are reported here.

SPUTA

All watery specimens of sputa were centrifuged for twenty minutes, the supernatant was discarded, and a smear was made from the residue. Smears were made from mucoid or mucopurulent specimens directly. The Ziehl-Neelsen method of staining was used. All specimens were examined microscopically and those which were negative after a careful search were digested and cultured.

*Methods of "Concentration"*—One part of sputum was digested with two to five parts of 4 per cent sodium hydroxide which had been sterilized by autoclaving. The amount of sodium hydroxide used depended on the character of the sputum. It is essential that the mucus and pus be dissolved. Fifteen cubic centimeter pyrex centrifuge tubes with rubber stoppers were used. The mixture was shaken by hand until it was completely in solution. The tubes were then placed in the incubator at 37° C for thirty minutes and subsequently centrifuged at high speed for twenty to twenty-five minutes. The supernatant was removed† and the sediment neutralized with hydrochloric acid using bromthymol blue as an indicator. A 2.5 N solution of hydrochloric acid was used to bring the pH to approximate neutrality. A 3 per cent (3 cc concentrated hydrochloric acid to 97 cc water) solution of hydrochloric acid was then used for final titration (pH 7.0). Centrifugation for twenty minutes was again necessary to pack the sediment so that a smear of the digested material could be made.

A certain number of specimens were concentrated by the ferric chloride flocculation method as described by Hanks in 1937<sup>1</sup>. Since the time of this publication, Dr Hanks has found flocculation with alum superior to that with ferric chloride<sup>2</sup>. We have not, as yet, tried this latter method. We agree with

\*From the Bureau of Bacteriology, Maryland State Department of Health, Baltimore.

Received for publication May 24, 1938.

†At the present time the middle portion of the supernatant is discarded and the top serum and sediment resuspended in about 0.2 cc of the supernatant and planted.

Dr. Hanks that centrifugation alone of the sodium hydroxide digests is not sufficient to collect all the tubercle bacilli and we anticipate carrying on further work on this problem.

All smears of concentrated material were carefully examined. About five to seven minutes were spent on each slide.

*Method of Culture.*—Petragnani's medium was used in screw-top tubes with cork washers. This medium had been found rather generally, as well as in the experience of this laboratory, to be superior to most other media. The screw-top tube has been found to be the simplest way to prevent drying of the culture medium. No appreciable drying of the media could be noted even with old cultures, yet these could be easily aerated by opening the tube each week at the time of examination.

The sediment remaining after the "concentrated" smear had been made was suspended in sterile saline\* and planted on one tube of the medium. The inoculated tubes were placed in the incubator in a horizontal position for twenty-four hours to allow the inoculum to adhere to the surface of the medium. They were then transferred to racks.

*Examination of Cultures.*—Cultures were inspected at approximately weekly intervals and smears were made of any colonies developing. All acid-fast bacilli isolated were inoculated into guinea pigs and their virulence determined before they were considered tubercle bacilli. Saprophytic acid-fast bacilli were quite frequently encountered; in young cultures they were impossible to differentiate from true tubercle bacilli, though their colonies could be readily differentiated, in most instances when older. All cultures were held three months before discarding.

*Results Obtained by Examination of Concentrated Smears.*—Smears of concentrated material were examined from 796 sputa negative on direct examination. Twenty-three, or 2.9 per cent, were found to be positive for acid-fast bacilli. Results according to the method or methods used in each case are given in Table I. All specimens were similarly treated with sodium hydroxide as described above.

TABLE I  
ACID-FAST BACILLI FOUND ON EXAMINATION OF "CONCENTRATED SMEARS"

METHOD OF COLLECTION FROM NaOH DIGEST	SPECIMENS EXAMINED	NUMBER POSITIVE	PER CENT POSITIVE
(a) Centrifugation only	484	15	3.1
(b) Ferric chloride and centrifugation	171	4	2.3
(c) Both methods (a) and (b) applied to equal portions of the same sputum	141	4*	2.8
Total	796	23	2.9

(b). \*Two were positive by both methods, one only by method (a) and one only by method (b).

Flocculation with ferric chloride did not increase the number of positives. The 23 positives obtained microscopically by "concentration" comprised 10 per cent of the total number of positive specimens obtained during the year. Cultures of these 23 specimens yielded typical tubercle bacilli in 17 instances, 4

\*See footnote † page 539.

were negative, and 2 unsatisfactory due to contamination. It seems likely that the acid fast bacilli found in 4 of the 6 negative or unsatisfactory specimens were tubercle bacilli since typical tubercle bacilli were recovered in other specimens from these individuals. Rarely acid fast bacilli have been found microscopically in concentrated material which on culture yielded only saprophytic organisms. One case (not included in Table I) was particularly interesting as a few acid fast bacilli were found in 2 of 3 specimens from the same person taken on different days. Only saprophytic acid fast bacilli were cultivated from all three specimens.

*Results Obtained by Culturing Microscopically Negative Sputa*—A total of 553 sputa found to be negative for acid fast bacilli on microscopic examination of smears made directly and after concentration were cultured. From 25, or 4.5 per cent, tubercle bacilli were isolated. Sixty one, or 11.0 per cent, were unsatisfactory due to contamination. Subtracting these unsatisfactory cultures from the total, the percentage of positives is increased to 5.0. The results by the different methods used for collection of the bacilli from the digested material are given in Table II.

TABLE II

TUBERCLE BACILLI OBTAINED BY CULTURING MICROSCOPICALLY NEGATIVE SPUTA

METHOD OF COLLECTION FROM NON DIGEST	POSITIVE		NEGATIVE	UNSATISFAC TORY	TOTAL
	NUMBER	PER CENT			
(a) Centrifugation only	10	3.98	200	32	251
(b) Ferric chloride and centrifugation	2	1.2	139	26	167
(c) Both methods (a) and (b) applied to equal portions of the same sputum	13*	9.4	121	3	137
Total	25	4.5	460	61	553

\*Eleven of these were positive by both methods. 2 were positive by the method where centrifugation alone was used for collection of organisms.

One hundred and thirty seven of the above sputa were divided, and equal portions were digested and "concentrated" by each method. These specimens were selected, as far as was possible, because of suggestive case histories. Tubercle bacilli were isolated from 13 (9.4 per cent) when only centrifugation was used, and from 11 (8.0 per cent) when ferric chloride and centrifugation were used. Statistically there is no significant difference in the results obtained by the two methods on the same specimens. Of 251 sputa which were digested and "concentrated" by centrifugation practically 4.0 per cent yielded positive cultures, while of the 167 which were digested and "concentrated" by flocculation with ferric chloride and centrifugation, 2, or 1.2 per cent, positive cultures were obtained. In our hands, flocculation with ferric chloride has not proved of value.

Eighteen and two tenths (18.2) per cent of the total number of positive sputa for the year were obtained by either the "concentrated smear" or by culture. Such an increase in the number of positives would seem to justify these examinations. Should the pressure of work be such that both examinations cannot be made, the culture method alone is indicated by these results. Using the culture method alone, however, 6 of the specimens positive on "concentrated



smear" would have been missed since these did not grow out on culture. The culture method gave 40, or 16.4 per cent, of the total number of positives.

### BODY FLUIDS

*Method of Treatment.*—Preliminary cultures were made on all specimens of spinal and pleural fluid, pus, and urine sent in for examination for tubercle bacilli. If these were negative after twenty-four hours' incubation, two or three tubes of Petraghani's medium and two guinea pigs were inoculated with approximately equal portions of the specimen. The ground clot and sediment were used. Specimens which were contaminated or which contained a large amount of pus were digested by the method used for sputum.

Cultures were examined as previously described. Some of the positives were found during the second week of incubation, but most of them became visible during the third or fourth week. As soon as the cultures were found to be positive, the guinea pigs were examined for enlarged inguinal glands. If these were present and the animals had been inoculated four weeks, one was sacrificed, otherwise the guinea pigs were held six weeks. At this time one was killed and autopsied. The other animal was held three months before sacrificing.

*Results of Culture and Animal Inoculations.*—Cultures and animal inoculations were made on 84 body fluids. The results are presented in tabular form (Table III).

TABLE III  
BODY FLUIDS EXAMINED FOR TUBERCLE BACILLI

MATERIAL	MICROSCOPIC			CULTURE				GUINEA PIG				TOTAL SPECIMENS
	+	% +	-	+	% +	-	Uns.	+	% +	-	Uns.	
Pleural fluid	9	17.6	42	19	37.2	30	2	13	25.5	33	5	51
Spinal fluid	2	13.3	13	5	33.3	10	0	4	26.6	10	1	15
Urine	0	0.0	9	1	11.1	8	0	0	0.0	8	1	9
Pus	1	12.5	7	3	37.5	5	0	3	37.5	5	0	8
Knee fluid	0	0.0	1	0	0.0	1	0	0	0.0	0	1	1
Total	12	14.3	72	28	33.3	54	2	20	23.8	56	8	84

Cultures were definitely superior to animal inoculations in this series. Twenty-eight, or 33.3 per cent, of the specimens were positive by culture, whereas only 20, or 23.8 per cent, were positive by animal inoculations. In no case were the guinea pigs positive when the cultures were negative.

### SUMMARY

Of 796 sputa negative on direct examination, the smears of 23, or 2.9 per cent, were positive after concentration. Ten per cent of the total number of positive specimens for the year were obtained by this method.

Cultures of tubercle bacilli were obtained from 25, or 4.5 per cent, of 555 microscopically negative sputa.

Eighteen and two-tenths per cent of the total number of positive sputa for the year were obtained either by concentrated smear or by culture.

All acid-fast bacilli isolated by culture must be tested for virulence to the guinea pig before they may safely be considered tubercle bacilli.

Cultures proved superior to animal inoculations when these methods were run in parallel on a series of 84 body fluids

The culture method used was simple and was quite practical for use in a public health laboratory

Acknowledgment is made of the technical assistance of Mr. John Krusinski and Miss Katherine Cunningham

#### REFERENCES

- 1 Hanks J, and Clark, H. A Method for Studying the Concentration of Tubercle Bacilli From Sputum and Body Fluids. *J. Bact.* 33: 57, 1937
- 2 Hanks J, Clark, H, and Feldman, H. Concentration of Tubercle Bacilli From Sputum by Chemical Flocculation Methods, *J. LAB. & Clin. Med.* 23: 736, 1938

---

## THE STANDARD FOR COMPARING THE VITAL CAPACITY OF SUBJECTS OF DIFFERENT SIZE AND A CHART FOR PRACTICAL USE\*

---

DAYTON J. EDWARDS AND MAY G. WILSON NEW YORK N. Y.

---

**A** DETERMINATION of the volume of air that a given subject can forcibly exhale is of little significance when detached from all other considerations of body build, since the general stature of the individual predetermines to a large extent thoracic size, to which this particular measurement of lung volume must be closely related. A primary consideration therefore in all evaluations of vital capacity is the other estimate of body build that most closely follows the normal difference in lung capacity, as it is by this means that a standard is made available for the comparison of the deviations resulting from disease.

In 1922 we made a study on children, ranging in age from 6 to 16, of the correlation between vital capacity and standing height, body weight, and body surface area.<sup>1</sup> The results of this analysis showed that for individuals in this size range, the surface area is slightly the more favorable index of body stature for reference of the vital capacity measurements, therefore, the practice was adopted of expressing this component of lung volume as a ratio of unit body surface area—the vital capacity surface area ( $VC/SA$ ) ratio. This technique has been in constant use for many years and it has been applied to a wide variety of patients with results indicative of a high degree of predictability in the evaluation of functional deficiencies of cardiac origin. A test of this kind, however, which depends on a comparison of one variable with another is useful in a practical way only in instances in which the factors tending to modify one component exert relatively small effects on the other. The  $VC/SA$  ratio appears to fulfill these conditions satisfactorily although the possibility exists that a disturbance which alters vital capacity if long continued, may modify the surface area size relationship in individuals under the age of maturity and thereby diminish the significance of this lung volume measurement as a clinical test.

\*From the Departments of Physiology and Pediatrics, Cornell University Medical College and the New York Hospital.  
Received for publication May 25, 1938.

In a similar way also the comparison of two variables for the purpose of detecting an abnormal condition implies a degree of uniformity between the factors over the range of normal measurements. Viewed in this light, our earlier studies<sup>1</sup> showed that the V.C./S.A. ratio was slightly less for subjects of smaller surface area size. This fact is well portrayed in Fig. 1, which is based on the original data, together with 70 additional subjects included in the larger body size groups.

At the time the original data were gathered on the vital capacity of normal subjects we were disposed to neglect the progressive decrease in the ratio for smaller subjects and to adopt as a standard for the normal individual in this size range the arithmetic mean of the 360 instances in the series. A chart was constructed on the basis of the single mean V.C./S.A. ratio,<sup>2</sup> and much valuable information has been obtained concerning heart disease in children by the use of this device. It is obvious, however, that an evaluation of a small subject present-

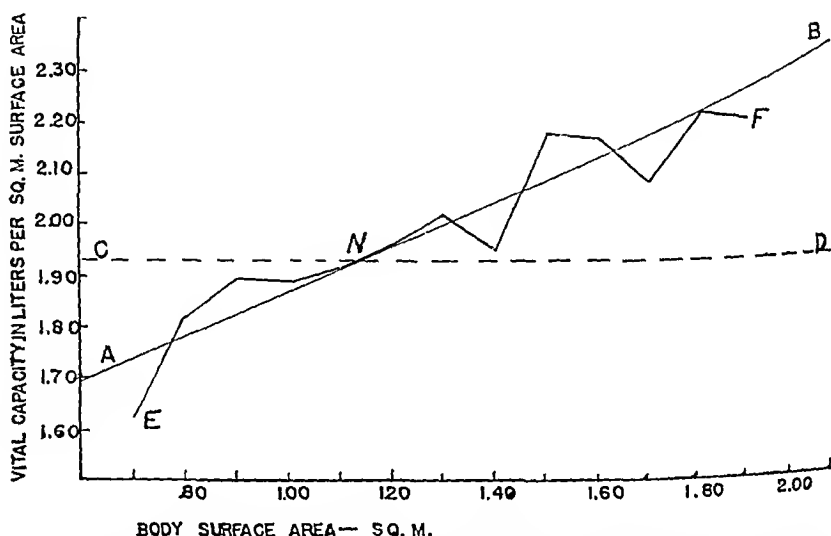


Fig. 1.—The graph E-F represents the vital capacity of 430 normal subjects reduced to a unit body surface area and arranged according to surface area size groups. The line A-B indicates the trend of the curve, and C-D the mean of the variations.

ing a subnormal vital capacity measurement tends to place him by this standard below par, whereas, for large subjects a primary deficiency becomes less evident on this scale. Fig. 1 illustrates this point very well by the dotted line C-D, which is placed according to the standard used in the former chart of 1.93 liters per sq. m. surface area, the locus being in the size group 1.10 to 1.19 sq. m. and designated N on the line C-D.

It has become increasingly evident that a correction for the size difference is desirable; therefore, a new chart has been drawn, which is presented in Fig. 2.

The basis of this chart likewise is in the material contained in Fig. 1, but the solid line A-B, drawn through a majority of the points plotted for the curve E-F, is adopted as the standard of reference. The intersection of A-B with each size group ordinate gives a regular ascending series of values for the V.C./S.A. ratio. This series represents, we believe, a closer approximation to the true

normal of the different size groups. Transposing these data into actual figures, we have in Table I the predicted vital capacity for each of the surface area size groups.

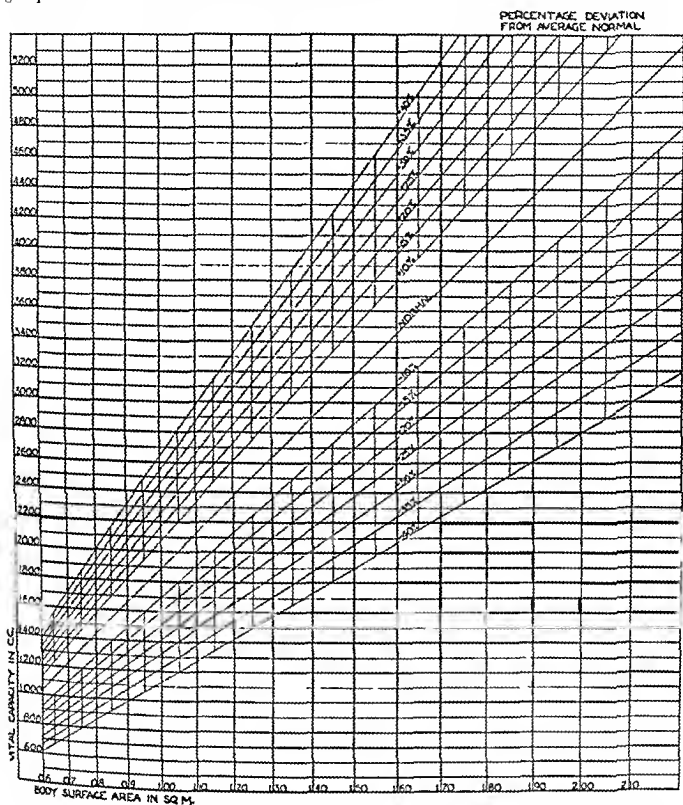


Fig. 2—A method for estimating the relationship of the vital capacity-surface area (V.C./S.A.) ratio from subjects of different size to a normal value obtained from the data of Fig. 1.

In arranging this material into a chart for practical use we have plotted vital capacity against surface area on a semilogarithmic scale, which makes possible straight line relationships and also permits dispensing entirely with definite values expressing the V.C./S.A. ratio as employed in the former chart.

The principal oblique line designated "normal" in the chart occupies the position indicated by the data contained in Table I. The remaining oblique lines occupy positions that denote definite percentage relationships to the normal, and they enable the user to determine directly the extent a given observation deviates from the expected value.

Although the data in Fig. 1 do not carry beyond the surface area size group of 1.8 to 1.9 sq. m., the chart has been extended to the 2.1 size group on the basis of the trend of the data. It is possible that this may be an unwarranted prediction, but some observations tend to support it.

TABLE I  
THE AVERAGE NORMAL VITAL CAPACITY FOR DIFFERENT SURFACE AREA SIZES

BODY SURFACE AREA SQ. M.	VITAL CAPACITY C.C.
0.6	1026
0.7	1228
0.8	1440
0.9	1660
1.0	1890
1.1	2128
1.2	2376
1.3	2632
1.4	2898
1.5	3172
1.6	3400
1.7	3748
1.8	4050
1.9	4360
2.0	4680
2.1	5008
2.2	5346

The primary advantage of this chart over the former one is that it gives readings at the extremes of the size range which conform more closely to our original data on normal subjects and to the results obtained in practical experience on patients. The original of this revised chart was drawn to the scale of 45 by 50 cm., but for practical use it is reduced to approximately 19 by 21 cm., which affords fair sized spacing for interpolating the fractional measurements.

#### REFERENCES

1. Edwards, D. J., and Wilson, M. G.: An Analysis of Some of the Factors of Variability in the Vital Capacity Measurements of Children, *Arch. Int. Med.* 30: 638, 1922
2. Wilson, M. G., and Edwards, D. J.: Diagnostic Value of Determining Vital Capacity of Lungs of Children, *J. A. M. A.* 78: 1107, 1922.

## A SIMPLE AND ACCURATE HEMATOCRIT TUBE\*

A T MILLER, JR., NORTHVILLE MICH

THE hematocrit tubes in current use leave much to be desired with respect to cost, accuracy, and amount of blood required. The tube to be described below requires less than 0.5 cc of blood, is free from error caused by shrinkage of cells resulting from loss of carbon dioxide during centrifugation and can be made from common laboratory materials.

Capillary tubing of uniform bore (1 to 2 mm) is cut into 12 cm lengths. The uniformity of bore of the capillary tubing may be determined by filling the tube with mercury and weighing successive 1 cm columns of the mercury delivered into a weighing bottle. Most of the capillary tubing now on the market

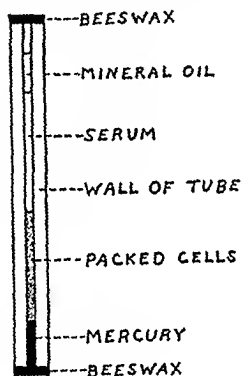


Fig 1

is quite uniform in bore. By means of a rubber tube similar to that used for blood pipettes, successive columns of mineral oil (1 cm), blood (8 to 10 cm), and mercury (1 cm) are sucked into the tube. The ends of the tube are quickly sealed by brushing with melted beeswax, reinforced by a heavy rubber band, which is prevented from slipping off by strips of adhesive placed over the ends of the tube at right angles to the rubber band.

Forty five minutes centrifugation at 3,000 r.p.m. packs the cells to constant volume. The total height of the blood column and the height of the column of packed cells are read off with a millimeter scale and the cell volume calculated. Liquid menisci at each point make the readings easy and accurate. Extensive use of this method over a period of three or four years, on both normal and pathologic bloods, has indicated that the maximum variation in quadruplicate determinations on a single sample of blood is consistently less than 0.5 per cent.

\*The William H. Maybury Sanatorium (Detroit Municipal Tuberculosis Sanatorium) Northville

# THE DETERMINATION OF AMINO ACID NITROGEN IN BLOOD AND URINE\*

## A RAPID COLORIMETRIC METHOD

MELVILLE SAHYUN, M.A., PH.D.

WITH THE TECHNICAL ASSISTANCE OF M. GOODELL, B.S.  
DETROIT, MICH.

THE colorimetric determination of amino acid nitrogen in blood, urine, and protein-free solutions is based on the reaction of the amino acids with  $\beta$ -naphthoquinone sulfonic acid in alkaline solution. This reaction was first discovered by Folin<sup>1</sup> who made use of it in his well-known method of amino acid nitrogen determination. Folin observed that the reaction between the dye and the amino acids required from nineteen to twenty-four hours in the dark to come to completion before the samples were suitable for comparison against an amino acid standard. Later Danielson<sup>2</sup> suggested a modification of Folin's method. He adopted a mixed amino acids standard, consisting of glycine and glutamic acid, which produces a color that matches very nearly that produced by tungstic acid blood filtrates. However, no attempt was made to shorten the time required for the completion of the analysis.

In this investigation we propose a very rapid colorimetric method for the determination of amino acid nitrogen in blood, urine, and protein-free solutions. Owing to the rapidity and sensitivity of the method, we are also in a position to propose a microquantitative method that will permit the quantitative estimation of about 0.005 mg. amino acid nitrogen in a given sample. Thus the filtrate obtained from 0.1 c.c. of blood is sufficient for the micro-determination.

### GENERAL CONSIDERATION

We have found that upon heating, amino acids react very readily with  $\beta$ -naphthoquinone sulfonic acid in an alkaline solution. Thus it is possible to eliminate the long period of waiting for the completion of the reaction and to minimize the danger of reading before the reaction has gone to completion. In order to determine whether or not this rapid reaction is quantitative, we investigated from 12 to 14 different pure amino acids. In this connection it is important to note that the depth of color is not proportional to the total amount of nitrogen, but corresponds to one amino group in the molecule. Thus it must be borne in mind that the determination of amino nitrogen of an amino acids mixture by the use of  $\beta$ -naphthoquinone sulfonic acid is not indicative of either the total nitrogen or even the total amino nitrogen of the

\*From the Biochemical Research Laboratory, Frederick Stearns and Co., Detroit, Mich.  
Received for publication, June 9, 1938.

amino acids In the simple amino acids, where one amino group is present in the molecule and where the total nitrogen corresponds to the amino nitrogen, the complex formed between the dye and the amino acid represents the total amount of nitrogen In certain amino acids where more than one amino group is present in the molecule, such as arginine, histidine, lysine, and tryptophane, the color formed in comparison with a standard such as glycine or alanine represents the nitrogen of only one amino group in the molecule

TABLE I  
DETERMINATION OF PURE AMINO ACID NITROGEN BY THE PROPOSED METHOD

AMINO ACIDS	TOTAL AMOUNT OF $\text{NH}_2\text{N}$	
	THEORETICAL	ACTUAL
Alanine	15.7	16.2
Glycine	21.0	20.8
Glutamic acid	8.8	8.5
Cystine	9.5	9.4
Histidine (HCl)N/.	6.1	5.8
Isoleucine	9.0	9.3
Leucine	9.0	9.1
Tryptophane N/2	7.7	7.8
Tyrosine	7.7	7.5

The mixed glycine glutamic acid standard was used. Proline, valine, phenylalanine, lysine, and arginine were also tried separately. The final color obtained did not match that of the standard. No efforts were made to read the samples.

#### AMINO ACID DETERMINATION IN BLOOD

In order to prepare suitable blood filtrates we recommend the following procedures, depending on whether total or plasma amino acids need be determined

1 Fohn and Wu's<sup>4</sup> method which consists of precipitating the blood proteins with 10 per cent sodium tungstate and  $\frac{2}{3}$  normal sulfuric acid yields excellent filtrates suitable for total amino acids determination

2 Fohn's<sup>4</sup> method for unlaked blood protein precipitation yields filtrates suitable for plasma amino acid nitrogen only. Owing to the presence of sulfate in the filtrate, the final color is somewhat bleached and does not match the standard unless sodium sulfate is added to the standard in amount similar to that which remains in the filtrate

3 Somogyi's<sup>5</sup> method which consists of precipitating blood proteins with zinc hydroxide yields excellent filtrates for amino acids determination. The copper sulfate method<sup>6</sup> is not suitable

4 Benedict's<sup>7</sup> method is also satisfactory

Blood was withdrawn from rabbits that had been fasted for twenty four hours. Each sample of blood was divided into two parts. One served as a control, to the other a certain amount of amino acid was added. The selection of these amino acids was carefully made in order to determine (1) whether or not such highly insoluble amino acids as tyrosine, cystine, leucine, and tryptophane were completely recovered in the filtrates of tungstic acid blood protein precipitation, (2) if the amount added could be quantitatively estimated by the proposed method, and (3) if excess of one amino acid would cause any difficulty in color comparison



The results are found in Table II. The data presented in the fourth column represent the difference between the figures recorded in the second and third columns.

TABLE II

ADDITION OF PURE AMINO ACIDS TO BLOOD AND  $\text{NH}_2\text{-N}_2$  DETERMINATION BY THE PROPOSED METHOD

AMINO ACID USED	SAMPLE NO.	$\text{NH}_2\text{-N}_2$ : MG./100 C.C. BLOOD			
		CONTROL	TOTAL	AMINO ACIDS	
				FOUND	ADDED
		mg.	mg.	mg.	mg.
Alanine	1	8.9	20.4	11.4	11.4
	2	10.5	21.6	11.1	
	3	9.6	21.4	11.8	
Histidine	4	8.9	15.0	6.1	6.2
	5	10.0	16.2	6.2	
	6	11.1	17.7	6.5	
Tryptophane	7	9.8	16.4	6.6	6.9
	8	9.9	16.7	6.8	
	9	9.5	16.2	6.7	
Cystine	10	11.1	20.8	9.7	9.5
	11	11.7	22.2	10.5	
	12	10.3	20.0	9.7	
Tyrosine	13	11.1	19.4	8.3	7.7
	14	11.7	20.4	8.7	
	15	10.5	19.0	8.5	
Arginine	16	9.8	15.7	5.9	5.9
	17	9.9	15.6	5.7	
	18	9.9	15.7	5.6	
Leucine	19	11.1	22.2	11.1	10.7
	20	10.4	21.4	11.0	
	21	9.1	19.6	10.5	

*Reagents.*—*Alkali:* 0.1 normal sodium hydroxide.

*Sodium Borate:* 2 per cent solution.

*Phenolphthalein:* 0.25 per cent solution.

*$\beta$ -Naphthoquinone Sulfonic Acid Solution:* 0.5 per cent solution, prepared freshly before using. Do not use a solution that has stood for more than fifteen or twenty minutes.

*Special Acetic Acid Acetate Reagent:* Dilute 100 c.c. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate.

*Sodium Thiosulfate:*  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  4 per cent solution.

*Standards:* If a single amino acid standard is desired alanine is suggested, provided a pure sample can be secured; however, a mixed standard of glycine and glutamic acid, as suggested by Danielson,<sup>2</sup> is preferred.

*Stock Standard Solutions:* Samples of glycine and glutamic acid are dried to constant weight over sulfuric acid in vacuum. Accurately weigh 267.5 mg. of glycine and dissolve in 0.07 normal hydrochloric acid containing 0.2 per cent sodium benzoate as preservative. The resulting solution is made up to 500 c.c. in a volumetric flask, using 0.07 normal hydrochloric acid—0.2 per cent sodium benzoate as diluting fluid. A stock solution of glutamic acid is prepared in the same way, using 525 mg. glutamic acid to 500 c.c. of 0.07 normal hydrochloric acid in 0.2 per cent sodium benzoate. The stock solutions thus prepared will each contain 0.1 mg. of amino acid nitrogen per cubic centimeter and will keep indefinitely.

*Mixed Standard 0.01 mg  $\text{NH}_2\text{N}$ .* Introduce 5 cc of the stock glycine standard and 5 cc of the stock glutamic acid standard into a 100 cc volumetric flask. Make up to volume with distilled water, mix thoroughly, add a few drops of chloroform. Keep in the refrigerator when not in use. It is preferable to prepare the dilute standards once a week.

*Procedure*—Into a graduated 25 cc or 30 cc test tube, introduce 3 cc of the filtrate to contain about 0.03 mg amino nitrogen. Simultaneously measure 3 cc of the standard amino acid solution (0.03 mg  $\text{NH}_2\text{N}_2$ ) into another tube. It is desirable to have the test tubes of uniform thickness. Add one drop of phenolphthalein to each tube, and while shaking the tube, add 1 drop at a time of 0.1 normal sodium hydroxide until 1 drop brings about a permanent pink color. Add 1 cc of sodium borate, followed by 2 cc of freshly prepared  $\beta$ -naphthoquinone sulfonic acid, mix by gently rotating the tubes and adjust the samples to equal volume by the addition of distilled water. It is desirable not to increase the volume any more than is necessary. Immerse in a boiling water bath for three minutes, remove and cool to room temperature in a stream of running water. To each tube first add 2 cc of the acetic acid acetate reagent, mix and then add 2 cc of 4 per cent sodium thiosulfate. Dilute to the 25 cc mark, mix, and make color comparison.

In selecting the standard for comparison, always select the one that most closely approaches the unknown in its concentration, as too wide a variation will lead to greater error in colorimetric measurement. In our experience we consider from 15 to 28 as reliable readings when the standard is set at 20. If the reading is stronger than 15 or weaker than 28 either the unknown is read against the next standard that gives a closer match, or the experiment is repeated with another suitable dilution.

#### DETERMINATION OF THE AMINO ACID NITROGEN IN URINE

As Folin<sup>8</sup> has pointed out, and as we have observed in this laboratory, it is necessary to remove all traces of ammonia from the urine before proceeding with the quantitative estimation of urinary amino acid nitrogen, since ammonia gives a strong color reaction with  $\beta$ -naphthoquinone sulfonic acid.

Owing to the fluctuations of urinary amino acid nitrogen, it is difficult to tell how much urine should be taken. It is, therefore, advisable to make simultaneously two dilutions of the urine in the ratios 1:10 and 1:20.

The process is as follows. Introduce 5 cc of urine into a 50 cc and a 100 cc volumetric flask and dilute to volume. Mix well and transfer to Erlenmeyer flasks. Add 2 or 3 gm of permittit, and shake gently for five minutes. Decant into clean flasks and again add the same amount of permittit, shake, and let stand for ten minutes before filtering. It is advisable at this point to test 1 cc of the filtrate with Nessler's solution to ascertain the complete absence of ammonia.

From the clear, ammonia free diluted urine, transfer 2 cc and 3 cc samples into graduated 25 cc or 30 cc test tubes. Simultaneously prepare two standards from the 0.01 mg amino acids standard previously described, one containing 0.03 mg and another 0.05 mg. Follow the directions given for blood amino acid determination.

*Micromethod Reagents.*—*Dilute Tungstic Acid Solution:* Transfer 20 c.c. of 10 per cent sodium tungstate to a liter volumetric flask, dilute to about 800 c.c. Add with shaking 20 c.c. of  $\frac{2}{3}$  normal sulfuric acid and dilute to volume.<sup>9</sup>

*Standard:*  $\text{NH}_2\text{-N}_2$  0.001 mg. This is prepared by accurately introducing 5 c.c. of the 0.01 mg. standard amino acid mixture into a 50 c.c. volumetric flask and diluting to volume.

The other reagents are the same as those used for the determination of amino acids in the blood.

TABLE III

MACRO- AND MICRODETERMINATION OF  $\text{NH}_2\text{-N}_2$  IN BLOOD

Blood of rabbits previously fasted for twenty-four hours was used. For the macromethod Folin-Wu's filtrate was used; for the micromethod Folin's and Somogyi's filtrates were used simultaneously.

SAMPLE	$\text{NH}_2\text{-N}_2$ : MG./100 C.C. BLOOD		
	MACROMETHOD	MICROMETHOD	
	FOLIN-WU FILTRATE	FOLIN FILTRATE	SOMOGYI FILTRATE
	mg.	mg.	mg.
1	9.7	9.0	11.1
2	9.7	9.1	9.5
3	10.5	9.8	9.3
4	11.7	---	11.1
5	8.7	9.1	9.1
6	8.9	9.5	8.7
7	9.3	9.5	9.8
8	9.2	9.1	9.1
9	8.7	8.7	10.0
10	8.6	8.7	9.5

TABLE IV

## MACRO- AND MICRODETERMINATION OF AMINO ACID NITROGEN OF BLOOD OF WELL-FED RABBITS BY THE PROPOSED METHOD

SAMPLE	$\text{NH}_2\text{-N}_2$ : MG./100 C.C. BLOOD	
	MACROMETHOD	MICROMETHOD
	mg.	mg.
1	15.3	15.1
2	15.4	14.0
3	15.0	15.1
4	15.0	15.3
5	15.2	15.7
6	14.5	14.3
7	15.1	14.3
8	13.7	13.1
9	15.4	15.5
10	16.3	15.7
11	14.5	13.8
12	15.9	14.6
13	15.4	14.6
14	14.5	14.1
15	15.9	14.8

Using a calibrated micropipette, introduce 0.1 c.c. of blood into 10 c.c. of the special dilute tungstic acid solution in a 15 c.c. centrifuge tube. Mix well, let stand for about five minutes, and centrifuge.

Should it be desired to use a different micromethod for the precipitation of blood proteins, both Somogyi's<sup>5</sup> and Benedict's<sup>10</sup> methods yield good filtrates that are suitable for the microdetermination of amino acids.

TABLE I

AMINO ACID DETERMINATION IN URINE AND THE RECOVERY OF KNOWN AMOUNTS OF ADDED PURE AMINO ACIDS

SAMPLE	AMINO ACID N		NH <sub>4</sub> N <sub>2</sub> MG/100 CC URINE	
	NATURE	ADDED	TOTAL FOUND	URINE NH <sub>4</sub> N LESS AMOUNT ADDED
1	None	mg	mg	mg
	Glycine glutamic	-	18.0	18.0
	Glycine	20	39.0	19.0
	Glutamic	10	30.0	20.0
2	None	10	30.2	20.2
	Glycine	-	30.2	0.2
	Glutamic	10	30.1	20.1
	None	10	0	20
3	None	-	20.0	20.0
	Glycine	10	30.0	20.0
	Glutamic	10	30.0	20.0
	None	10	30.0	20.0
4	None	-	14.0	14.0
	Glycine glutamic	20	36.0	16.0
	Glycine	10	25.0	15.0
	Glutamic	10	24.0	14.0

**Procedure**—Into a graduated test tube, introduce 5 cc of the filtrate and simultaneously measure 5 cc of the microstandard (0.005 mg). Add to each 1 drop of phenolphthalein, neutralize to a definite pink with 0.1 normal sodium hydroxide, employing the same precautions described in the macromethod. Add 1 cc of 2 per cent sodium borate and 1 cc of the freshly prepared  $\beta$ -naphthoquinone sulfonic acid. Immerse in a boiling water bath for three minutes, cool, add 1 cc of acetic acid acetate reagent followed by 1 cc of 4 per cent sodium thiosulfate. Dilute to the 15 cc mark, mix, and compare colors.

## SUMMARY

A rapid colorimetric method for the determination of amino acid nitrogen in blood and in urine is described. Details for the macro and micro technique are also given. The results obtained by its use are self explanatory.

## REFERENCES

1. Fohn, O. A New Colorimetric Method for the Determination of the Amino Acid Nitrogen in Blood, *J Biol Chem* 51 377 1922
2. Danielson, I. S. Amino Acid Nitrogen in Blood and Its Determination *J Biol Chem* 101 505 1933
3. Fohn, O., and Wu, H. A System of Blood Analysis *J Biol Chem* 38 81 1919
4. Fohn, O. Unclotted Blood as a Basis for Blood Analysis *J Biol Chem* 86 173 1930
5. Somogyi, M. A Method for the Preparation of Blood Filtrates for the Determination of Sugar, *J Biol Chem* 86 655 1930
6. Somogyi, M. The Use of Copper and Iron Salts for the Deproteinization of Blood *J Biol Chem* 90 725 1931
7. Benedict, S. R. The Analysis of Whole Blood I The Precipitation of the Proteins, *J Biol Chem* 92 135, 1931
8. Fohn, O. A Colorimetric Determination of the Amino Acid Nitrogen in Normal Urine, *J Biol Chem* 51 393 1922
9. Fohn, O., and Malmros, H. An Improved Form of Fohn's Micromethod for Blood Sugar Determination, *J Biol Chem* 83 115, 1929
10. Benedict, S. R. The Analysis of Whole Blood II The Determination of Sugar and of Saccharoids (Nonfermentable Copper Reducing Substances), *J Biol Chem* 92 141, 1931

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**ASCHHEIM-ZONDEK, A Modification,** Walker, T. F., and Walker, D. V. N. J. A. M. A. 111: 1460, 1938.

1. The morning specimen of urine is collected.
2. A female white rat from 30 to 45 days old is injected with 1.5 c.c. of urine three times during the day.
3. Thirty hours after the first injection the animal is killed with chloroform and the ovaries are examined.

In positive cases the ovaries are enlarged and hyperemic and show hemorrhagic follicles.

**HODGKIN'S DISEASE, The Gordon Test for,** McNaught, J. B. J. A. M. A. 111: 1280, 1938.

In the author's series the Gordon test was positive in 10 of 13 cases of Hodgkin's disease (77 per cent) and negative in 35 of 37 control cases of various lymphadenopathies (95 per cent). These cases being included, the Gordon test has been reported positive in 70 per cent of 192 cases of Hodgkin's disease and negative in 98 per cent of 251 control cases.

Histologic sections of the lymph nodes eliciting the positive reactions showed numerous eosinophiles, while the nodes associated with the negative reactions contained very few or no eosinophiles.

Extracts of human tissues and leucocytes which were from patients who definitely did not have Hodgkin's disease, but which contained many eosinophiles, caused reactions in rabbits indistinguishable from those caused by the lymph nodes from patients with typical Hodgkin's disease. This supports the theory that Gordon's agent and Friedemann's agent are identical and are apparently derived from the eosinophile.

Histologic studies have already shown that eosinophiles are easily demonstrable in the glands of some 70 per cent of patients with Hodgkin's disease, and it is in the same percentage of cases that the Gordon test is positive. Apparently it is positive only by virtue of these cells.

The Gordon test is of no more practical value in the diagnosis of Hodgkin's disease than is the finding of eosinophiles in the lymph nodes.

**ADRENAL INSUFFICIENCY, Chloride, Sodium and Potassium, Concentrations of, in Urine and Blood, Diagnostic Significance of, in,** Cutler, H. H., Power, M. H., and Wilder, R. M. J. A. M. A. 111: 117, 1938.

Under standardized conditions as described, the concentration of either chloride or sodium in the urine of patients with Addison's disease has been diagnostically more significant than any other factor examined, giving more valuable information than the volume of the urine, the concentration of potassium in the urine, or the values or change in values during a fifty-two-hour period of restricted intake of sodium chloride, or the chloride, sodium or potassium of the blood plasma.

The diagnostic procedure suggested requires fewer days for completion, subjects the patient to less risk of collapse, and in most cases is quite as informative as the six-day period of restricted intake of salt heretofore resorted to for diagnostic purposes.

As the concentration of the chloride in the urine gives an important information as that of sodium, the analytic procedure demanded may be limited to analysis for urinary chloride. This represents a distinct advantage, because many clinics and hospitals are not equipped for analytic determinations of sodium or potassium.

The authors' conclusions are based on examinations of 17 patients with Addison's disease and 37 controls. The group of controls included 10 healthy persons and 27 patients with disease which apparently did not involve the adrenal glands.

The standardized procedure follows:

When patients with Addison's disease previously were receiving either adrenal cortex extract or extra salts of sodium, these were withheld on the day preceding the first day of the special examination. Longer periods of such preparation proved to be unnecessary.

On the first day of the examination, and thereafter until its close, a diet low in salt was served, which by calculation provided 0.95 gm. of chloride ion, 0.59 gm. of sodium, and 41 gm. of potassium. The fluid intake of the first day was not measured, but the free drinking of water was encouraged. On the afternoon of the first day extra potassium was given, as potassium citrate, in a dose representing 77 mg. of potassium per kilogram of body weight (42 mg. of potassium citrate per pound).

On the second day the intake of liquid was made to equal 40 cc. for each kilogram of body weight, and on the morning of this day the dose of potassium citrate was repeated.

On the third day 20 cc. of liquid per kilogram of body weight was given before 11 A.M. At 12 noon of this third day the examination ended except in those cases in which the period of deprivation of salt was extended in order to obtain additional evidence of the state of adrenocortical function. At the close of every examination, in all cases in which adrenal insufficiency was suspected, an intravenous injection was given of 1,000 cc. of sterile solution containing 50 gm. of d-glucose, 10 gm. of sodium chloride, 5 gm. of sodium citrate, and 20 cc. of an active preparation of cortical hormone.

Blood was drawn in an oiled syringe from the cubital vein at 8 A.M. of the second day of examination and at 10 A.M. of the third day. It was transferred to cooled 20 cc. hematocrit tubes containing heparin and oil, and the plasma was separated by means of a refrigerated centrifuge. Urine was collected in three periods: from 8 A.M. to 8 P.M. of the second day, from 8 P.M. to 8 A.M. of the third day, and from 8 A.M. to 12 noon of the third day.

**PNEUMONIA, Pneumococcus Studies in Liver Function in, Curphey, T. J., and Solomon, S.** *Am J M Sc* 196 348, 1938

Multiple methods embodying the study of pigment and carbohydrate metabolism have been utilized concurrently in a study of liver function in 80 cases of pneumococcus pneumonia.

The urobilinogen excretion was increased in the majority of both the fatal and recovered cases and markedly high values of excretion are twice as frequent in the fatal group. When serial observations were made from day to day in the course of this disease, the level of pigment excretion in the urine tended to fall in the recovered cases, but to remain persistently high or to rise in the fatal group.

The icterus index was elevated in both fatal and recovered cases in the early stages of pneumococcus pneumonia. There was a constant tendency to falling icterus index values in the cases that recovered and a tendency to remain high in the fatal cases.

There is decreased levulose tolerance in a high percentage of the recovered cases and in all of the fatal cases early in the disease. Serial observations in fatal cases show a progressive decrease in the ability to metabolize the sugar, whereas in the cases that recover, there is a progressive increase in levulose tolerance.

It is a further question whether daily determinations of fasting blood sugar levels do not provide definite information of similar significance as that afforded by levulose tolerance tests.

These observations stress the importance of serial determinations in any functional test in the course of the study of an acute infectious disease like pneumonia.

**SEDIMENTATION RATE of Red Blood Cells in Plasma, Pleural and Ascitic Fluids, Cohen, S., Faraci, P., and Pollak, B. S. Am. Rev. Tuberc. 38: 372, 1938.**

The technique of Kling was employed in obtaining comparative sedimentation curves of pleural and ascitic effusions and of the patient's own plasma. The material comprised a total of 61 cases in which 153 fluid sedimentation rate determinations were made and an equal number of blood readings. Pertinent additional laboratory tests were made on the aspirated fluids. The blood sedimentation rates (according to the modified Westergren method) on 50 apparently normal individuals were also determined.

The results of comparative sedimentation taken in groups were quite constant. This cannot be emphasized too strongly, for individual cases will show variations from the average findings. The latter do not represent absolute lines of cleavage which are always applicable in every instance. With these preliminary remarks, the authors note:

(a) The sedimentation of erythrocytes, in general, is rapid in inflammatory effusions having a high protein content, and low in fluids of noninflammatory origin having a low protein content. This confirms the conclusion of Garnier and Oumansky. Coincidentally, the former show a high comparative index which reaches its maximum with purulent tuberculous exudates and the latter a low index. "The comparative index reveals the share that the local process contributes to the general reaction." (Kling.)

(b) Using the patient's cells, the typical fluid sedimentation curves for the effusions studied were: (1) transudate: a horizontal line; (2) serous exudate: a diagonal line; (3) purulent exudate: a vertical line or curve.

(c) Again, considered as unit groups, there is noted some parallelism between the average specific gravity, the average quantitative protein, and the average type of fluid curve.

(d) Comparison of serial fluid curves may be employed as a laboratory adjunct to determine regression or progression of exudation in tuberculous pleuritis. However, clinical and radioscopic examinations still remain of paramount importance.

**VITAMIN C Deficiency, Intradermal Test for, Poucher, H. G., and Stubenrauch, C. H., Jr. J. A. M. A. 111: 302, 1938.**

The authors' results do not confirm the conclusions of Rotter or Portnoy and Wilkinson. The data indicate that the intradermal dye test, in its present form, cannot be relied upon to give satisfactory clinical information as to the cevitamic acid saturation in the individual case. Further study is necessary to determine whether the principle behind the test can be utilized clinically in assessing the state of vitamin C nutrition.

**Method and Technique.**—A solution containing 2 mg. of the powdered dye 2:6-dichlorophenolindophenol in 4.9 c.c. of distilled water was employed for the intracutaneous injections. To prepare this solution, the dye was dissolved in the proportion of 4 mg. of dye to 4.9 c.c. of distilled water. The solution was then passed through a Seitz filter for sterilization. A portion of the sterile solution was removed with a sterile pipette and titrated against a known solution of cevitamic acid (standardized against hundredth normal iodine solution). A solution of dye containing 2 mg. of 2:6-dichlorophenolindophenol in each 4.9 c.c. of water was also titrated against the known solution of cevitamic acid. Then the sterile filtrate was diluted with sterile water to its correct strength of 2 mg. of dye in 4.9 c.c. of distilled water. After two weeks, the solution was restandardized or a fresh solution was prepared.

The injections were made into the skin on the volar surface of the forearm. An area was chosen where there were no hairs or superficial veins. It was cleansed with ethyl alcohol and allowed to dry. Then an intradermal injection was made in the usual way. The dye was injected (0.01 c.c.) immediately beneath the epithelium. This resulted in a wheal about 2 mm. in diameter. The time of injection and the time of complete disappearance of the blue color were recorded. From two to eight injections were made in each subject, and an average of the disappearance times was taken as the correct value for that patient.

At the time the tests were made, 5 cc of blood was drawn from each subject and the ceritamic acid level was determined. The micro-method of Farmer and Abt was used in this procedure.

**SYPHILIS, Significance of Positive Kline Exclusion Test, Unconfirmed by Kolmer or Kahn Test, Myers, R. M., and Perry, C. A. J. A. M. A. 111 142, 1938**

It was shown that in definite instances the positive Kline exclusion test, unconfirmed by the Kolmer or the Kahn test, is significant of syphilitic infection.

It was further shown that physicians tend to accept without question the negative serologic report, whereas a doubtful report elicits further clinical consideration and serologic checkup.

No evidence was found to indicate that doubtful reports were interpreted as positive.

Therefore, a report giving some indication of the reaction of the very sensitive Kline exclusion test would seem to be an advantage to the patient and an aid to the physician.

**POLIOMYELITIS VIRUS in Human Stools, Trask, J. D., Vignee, A. J., and Paul, J. R. J. A. M. A. 111 11, 1938**

Poliomyelitis virus was found in four clinical cases of poliomyelitis from the nasopharynx in three cases, and three times from the stools in one case.

Human stool extract for inoculation into monkeys does not have to be injected intracerebrally in order to produce the experimental disease.

The attacks in all cases which yielded the virus were mild and nonparalytic. It is probable that some of the attacks might not have been regarded as examples of poliomyelitis without this finding.

The child who showed virus in the stools was ill for only three or four days, and yet the virus persisted in the feces for at least twenty-four days from the onset of this mild illness. It also remained viable for ten weeks in one of his stools which was kept in the refrigerator. Such facts suggest that during an epidemic of poliomyelitis these common, mild, and often unrecognized forms of the disease, may be responsible for a high degree of pollution of sewage with poliomyelitis virus.

**TISSUE Modification of Terry's Method of Rapid Sectioning for Soft Tissues, Hall, W. E. B. Arch. Path. 25 854, 1938**

A glass cover slip is cemented to each end of an ordinary glass slide, leaving an intervening depressed space, from 2 to 2.5 cm in width and 1 mm in depth. On this depressed portion is placed the tissue to be examined, including the fine fragments. If a large mass is to be examined, this is cut down to a thickness of about 0.5 cm by scissors, knife, or safety razor blade. Curettings are patted together to lie as a flattened but compact mass against the cover slip border. A slight amount of tap water (a drop or two) is added to produce a completely moistened tissue. The slide is then turned over. If the tissues have not been too much moistened, they remain adherent to the glass slide as before. If the fragment or fragments persist in falling off, they may be held in place by the counter pressure of a 3 inch (7.6 cm) section of a tongue depressor stick, the ends of the stick and the glass slide being gently maintained between the thumb and second finger of one hand. The surface of the slide now uppermost is sprayed with ethyl chloride until the tissue underneath is frozen to a depth of from 2 to 4 mm. Again reversing the slide, the tissue is shaved off by firm but delicate application of a sharp common razor blade or preferably a new safety razor blade. Deep or forceful sectioning will result in fragmentation or elevation of the frozen tissue mass. Freezing is readily repeated if there are signs of softening. The tissue shaved to a thickness of from 1 to 2 mm, is allowed to thaw out, after which polychrome methylene blue is applied for fifteen seconds. The excess of stain is then allowed to drain off, supplemented at times with an extra drop of tap water. The specimen is now covered with a cover slip and examined by transmitted light, a 60 watt bulb or stronger illumination being used as indicated.



For small fragments this method is superior to ordinary frozen section methods and has proved satisfactory with small and very soft tissues which could not be readily examined by the ordinary Terry-Hellwig technique. It allows excellent differentiation of tissues with polychrome methylene blue. This stain is sprayed on the tissue superficially from an atomizer. According to the last article by Terry, he described the use of a relatively simple sectioning apparatus to obtain sections of tissue of uniform thickness. In contrast to the staining time used by Terry and Hellwig, it has been found better to leave the stain on the surface of the tissue, spilling over onto the slide, for at least fifteen seconds but not for more than thirty. It is then allowed to drain gently off the slightly tipped slide without washing, which all too frequently destroys color differentiation of tissues and intensity of stain. Likewise, the light used should be of considerable strength, increasing with the thickness of the tissue, its darkness, and the amount of blood contained in it. Light of the intensity of sunlight has been found satisfactory. However, when the border of the tissue is the point of interest, with or without the freezing modification, it has been found that the lateral refraction glare from the transillumination may be obviated by a slight excess of stain gathered about the sides of the fragments under the cover slip. For this, the excess drop staining method is still followed. A little practice is required to determine the usual amount of water needed for satisfactory fixation of tissue to the glass slide, so as to allow satisfactory freezing without loss or separation of tissue fragments, unless the counter-pressure of a section of a tongue depressor is used. A simple metal clip may be readily prepared by which the glass slide may be held in position on the ordinary carbon dioxide freezing block, and the tissue frozen without inversion of the slide. All fragments, together with the preliminary shavings, are subsequently placed in a 10 per cent concentration of solution of formaldehyde U.S.P., to be subsequently embedded in paraffin and sectioned.

**FUNGI, A New Species of *Madurella*, Hanan, E. B., and Zurett, S. Arch. Dermat. & Syph. 37: 947, 1938.**

The new species of *Madurella* is defined as follows: *Madurella lackawanna*, 1936: The mycelium is white or smoky gray, with a white periphery in old cultures. The colony tends to be spherical, with a central mycelial zone, a spore zone, and a peripheral zone: Growth is successful only on Sabouraud's dextrose agar, dextrose agar, and glycerin agar when enriched with liver infusion. The hyphae vary in dimensions from 1 to 5 microns and are coarsely granular. Septa are rare in early cultures, but definite fine septa with smoky gray mycelium appear in later cultures at room temperature. Chlamydospores appear early in the spore zone. There are nodular organs, consisting of arthrospores with square-cut ends, in the peripheral mycelium at room temperature. The optimal medium is fresh liver or liver infusion-Sabouraud's dextrose agar. The optimal temperature is between 20° and 37° C. The organism digests milk proteins, but does not liquefy gelatin, ferment milk lactose, nor digest milk fat. Pigment formation is abundant, but blackening of the medium is more pronounced at room temperature, except on glycerin agar enriched with liver. There is little or no growth in fluid media. Animal inoculation gives negative results. The organism was isolated in a case of maduromyces with black grains in Lackawanna, N. Y.

**PREGNANCY, Skin Tests for, Lass, P. M., Enderle, E. S., and Kurzrok, R. Endocrinology 23: 71, 1938.**

Two hundred and fifty-four patients were tested for pregnancy by means of the Gillen-Gregg skin test PU extract. The error in diagnosis ranged from 7 to 64 per cent, depending upon the type of patient chosen for the test. One hundred and eight patients were tested by means of the Gruskin skin test for pregnancy (placental antigen). The error in diagnosis ranged from 20 to 52 per cent, depending upon the type of patient chosen for the test. Hence neither test is reliable, nor of any value in clinical medicine.

# The Journal of Laboratory and Clinical Medicine

VOL 24

MARCH, 1939

No 6

## CLINICAL AND EXPERIMENTAL

### SPECIFICITY OF PNEUMOCOCCUS TYPES BY QUELLUNG AND AGGLUTINATION REACTIONS\*

ARYLL NOBLE, A B, AND BERTHA C CAMERON, B S DETROIT, MICH

CULTURES representative of the 27 types of *Diplococcus pneumoniae*, as established by Georgia Cooper<sup>1</sup> - and formerly known as group IV, together with types I, II, and III, were isolated and antisera prepared in rabbits. With these sera and cultures, homologous and cross agglutination and Neufeld-Quellung reactions<sup>1</sup> have been studied.

#### METHODS AND MATERIALS

**Cultures**—Sixty-three cultures of *Diplococcus pneumoniae* were used in this study. The samples of sputum from which a large number of these strains were obtained were made available to us through the courtesy of members of the staff at the Receiving Hospital, Detroit, where each sputum had been typed by the Neufeld-Quellung test. Within a period of three months cultures representative of the 30 types of pneumococci had been isolated by the mouse method. In this group, material from 65 cases was cultured. From 53 of these, the pneumococcus type corresponding to the sputum diagnosis was recovered, from 12 cases some other type of pneumococcus, Pfeiffer's bacillus or streptococcus was isolated. We were unable to type one apparently typical pneumococcus.

A few additional strains were obtained from sputa and blood cultures from cases of pneumonia at the Henry Ford Hospital. Two or more strains of each type were obtained in all but five (XVII, XXI, XXIII, XXVII, and XXVIII).

All cultures are gram positive cocci usually pointed occurring in pairs and short chains of pairs. They are all soluble in bile, 42 promptly ferment milk, 21 show slow fermentation, or none at all.

\*From the Research Laboratories Parke Davis & Co. Detroit.  
Received for publication June 23 1938





## CROSS AGGLUTINATION REACTIONS WITH

TYPE SERA	CULTURE USED FOR SERUM	SUSPENSIONS OF DIPLO											
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
1	02444	*4443											
2	02445		4400						±				
3	02179			4421									
4	1170				4444				±				
5	1171					4410							
6	1223						4420						
7	1161							4300					
8	1176								4444				
9	1174									4441			
10	1220								±		4444		
11	1216								±			4443	
12	1206								±				4444
13	1202												
14	1168												
15	1228								±				
16	1186								±				
17	1183								±				
18	1217								±				
19	1222								±				
20	1173-1					±			±		±		
21	1213												
22	1159								±				
23	1229												
24	1192				±								
25	1207												
27	1233												
28	1194								±				
29	1219				±		±		±	±			
31	1237								±				
32	1214								±	±			
Controls		0	0	0	0	0	0	0	0	0	0	0	0

\*Four amounts of undiluted serum were used—0.05, 0.025, 0.01, and 0.005 c.c., corresponding to dilutions of 1:20, 1:40, 1:100, and 1:200.

4 = complete agglutination; 3 = marked; 2 = partial; 1 = slight; 0 = no agglutination. The blank spaces indicate negative results.

The cultures are maintained in rabbit blood (one part defibrinated blood to two parts saline, about 5 c.c. to a tube). Cultures are incubated at 37° C. for eighteen hours and stored at 4° C.

*Method of Producing Antisera.*—Rabbits, weighing about 5 pounds each, were used throughout. Antigens of each type were prepared by suspending the young growth from ascites agar in saline plus 0.5 per cent formalin. Each rabbit received one dose subcutaneously and then intravenous doses of increasing amounts twice weekly for from three to six weeks. At intervals the rabbits were sample bled from the ear and the serum tested for Quellung reaction and for agglutination. If a clear-cut reaction was not obtained, they were further treated.

The Quellung tests were made on either peritoneal fluid from mice injected with the specific culture or on rabbit blood culture. In either case formalin had been added. The agglutination titers ranged from 1:40 to 1:200 and sometimes higher. The serum from two or three rabbits on each type was pooled and preserved with 0.5 per cent phenol.

*Technique of the Quellung Reaction.*—Eighteen-hour cultures in rabbit blood preserved with about 0.5 per cent formalin were used for the Quellung



## CROSS AGGLUTINATION REACTI

TYPE SERA	SUSPENSIONS OF D													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI			
1	1234	1235-1	1238	1180	1163	1221	1181	1154	1162	1240	1241	1178	1239	1189
2	4443													
3		4311												
4			4432					211*						
5				4421										
6					4444	4443								
7						10000*								
8			3100				4463	4444		4443	4444	1100*		
9												4444		
10													4442	
11														4444
12														
13														
14														
15														
16														
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														
27														
28														
29														
30														
31														
32														
Controls	0	0	0	0	0	0	0	0	0	0	0	0	0	0

\*Tests repeated with same results.

Four amounts of undiluted serum were used—0.05, 0.025, 0.01, and 0.005 c.c., corresponding to dilutions of 1:20, 1:40, 1:100, and 1:200.

The blank spaces indicate negative results.

*Technique of the Agglutination Tests.*—Agglutination tests were made by a rapid macroscopic method,<sup>4</sup> using measured amounts of undiluted serum and, for suspensions, sediment from broth cultures.

The supernatant growth from a twenty-four-hour rabbit blood culture is used to inoculate tubes of veal broth (proteose peptone, pH 7.8-8.0) which are incubated eighteen hours. Approximately 0.5 per cent formalin is added and the cultures thoroughly mixed. They are then centrifuged, the broth decanted, and the organisms resuspended in broth and formalin. This suspension is thoroughly mixed and allowed to stand for two or three hours in a narrow tube to allow any clumps or debris to settle. The supernatant homogeneous suspension is then removed to a clean tube and diluted with broth and formalin to a density of 5 to 8 billion organisms per c.c.

*Test.*—One-half cubic centimeter of each type serum is pipetted to the bottom of small tubes (Kahn tubes), and 0.1 c.c. suspension of the culture being tested added to each. For the homologous type the serum amounts are 0.05, 0.025, 0.01, and 0.005 c.c. The racks carrying the tests are inclined to an angle of 45° and slowly shaken for two minutes, 0.5 c.c. saline added, and the results read.

### ICATE CULTURES ON CERTAIN TYPES

[illegible]

## EXPERIMENTAL

*Cross Quelling Tests*—Each type serum was tested against the 30 type cultures which were used for antiserum production. The results are contained in Table I. In all the tables Arabic numerals represent serum types and Roman numerals the culture types. It will be seen that with the exception of culture No. 1237, type XXXI, which was positive also in type 20 serum there were no cross reactions.

Among the cultures not used for serum production another strain of type XXXI (No 1231) was the only culture which showed cross reactions. This was also positive in type 20 serum, while a third culture (No 1236) was negative (Table II).

Therefore 61 out of 63 eultures gave type specific reactions by the Neufeld Quellung test under the conditions of the experiment

**Cross Agglutination Tests.**—Cross agglutination tests with the 30 cultures used for antisera in general showed little crossing (Table III). Types VIII and XVI gave doubtful reactions with the majority of sera at 0.05 cc. Whenever a doubtful or apparently inconsistent result was obtained the tests were repeated.

Among the cultures not used for serum production (Table IV) one type III culture (1238) showed partial agglutination in type 8 serum, one type VII (1162) crossed slightly with 3, one type IX (1178) slightly with 8, and one



type XXXI (1231) showed marked agglutination in types 13, 20, and 29. Culture 1181, which was typed as VI by the Neufeld reaction and checked several times, failed to agglutinate in any serum.

One culture (1218) which is morphologically a pneumococcus, ferments inulin, and is soluble in bile, has been consistently negative by Neufeld and shows some degree of agglutination in practically all type sera, but complete agglutination in none.

*Agglutination and Quellung Reaction With Types III and VIII.*—As there has been considerable mention in the literature concerning the crossing of types III and VIII, a few additional strains were tested, including a culture of type III labeled "R<sub>3</sub>" and one of type VIII labeled "Henrique," received from Georgia Cooper, February, 1934, and an old type III, received from Rockefeller Institute in 1922. The results are given in Table V.

TABLE V  
AGGLUTINATION AND QUELLUNG REACTIONS WITH TYPES III AND VIII

CULTURES	AGGLUTINATION								QUELLUNG	
	SERUM 3*				SERUM 8*				SERUM 3	SERUM 8
	0.05 C.C.	0.025 C.C.	0.01 C.C.	0.005 C.C.	0.05 C.C.	0.025 C.C.	0.01 C.C.	0.005 C.C.		
III Rockefeller	3	4	4	4	1	-	-	-	+	-
02179	4	4	2	1	-	-	-	-	+	-
1238	4	4	3	2	3	1	-	-	+	-
1299	3	4	4	4	1	1	-	-	+	-
1300	4	4	4	4	2	1	-	-	+	-
Cooper R <sub>3</sub>	4	4	4	4	3	1	-	-	+	-
VIII 1176	-	-	-	-	4	4	4	4	-	+
1240	-	-	-	-	4	4	4	3	-	+
1241	-	-	-	-	4	4	4	3	-	+
Cooper "Henrique"	-	-	-	-	4	4	4	2	-	+

\*Serum 3 was produced with culture 02179 and Serum 8 with culture 1176.

Five out of six strains of type III crossed slightly with type 8 serum by agglutination, and the five type VIII strains showed no crossing with serum 3, while type specific reactions were obtained with all by the Quellung test.

#### SUMMARY

Cultures of *Diplococcus pneumoniae*, representative of types I, II, and III, and the Cooper types IV through XXXII (except XXVI and XXX which are now grouped as VI and XV, respectively) were isolated and antisera prepared.

These 30 types were found to be remarkably specific by both agglutination and Quellung reactions, the latter being the more specific.

In contrast to the numerous unsuccessful attempts which have been made in the past to separate various species of bacteria into types, the classification of the pneumococci as originated by the late Georgia Cooper has been repeatedly confirmed.

Our appreciation is due to Dr. A. H. Price of the Receiving Hospital and Dr. F. W. Hartman of the Henry Ford Hospital for their kindness in furnishing us with the material from which the cultures were obtained.

#### REFERENCES

1. Cooper, G., Edwards, M., and Rosenstein, C.: J. Exper. Med. 49: 461, 1929.
2. Cooper, G., Rosenstein, C., Walter, A., and Peizer, L.: J. Exper. Med. 55: 531, 1932.
3. Sabin, A. B.: J. A. M. A. 100: 1584, 1933.
4. Noble, A.: J. Bact. 14: 287, 1927.

## EVALUATION OF H AND O ANTIGENS IN AGGLUTINATION TESTS FOR TYPHOID FEVER\*

LUCIE R. HAY, PH D., CATHERINE S. FLANN, PH D., AND C. ALFRED PERRY, SC D  
BALTIMORE MD

THE value of carefully standardized H and O typhoid antigens as proposed by Felix<sup>1</sup> over Widal's (1896) living antigen or the formalized antigen is generally accepted. However, there is still uncertainty as to (a) what constitutes diagnostic titers with H and O antigens, (b) the effect of antityphoid inoculations on the development and persistence of H and O agglutinins, and (c) the respective value of living and of H and O antigens in routine public health work.

Felix considered a serum titer of 1:200 or more with an O suspension as a positive agglutination reaction for typhoid fever. The titers which other investigators<sup>2-6</sup> have considered to be significant have, however, varied widely. Rarely<sup>7-13</sup> have O titers as high as 1:160 been found in serum of vaccinated individuals unless vaccination against typhoid has been very recent (within six months). Agglutination titers with the O antigen in normal unvaccinated persons have usually been low.<sup>4-11, 14-16</sup> Occasional high titers may have been due to such persons being carriers.

There is, therefore, a definite need for further clarification and evaluation of reactions with these antigens before they can be used to best advantage in routine diagnostic laboratory work.

### SELECTION AND PREPARATION OF ANTIGENS

The wide variation in the agglutination titers considered indicative of typhoid fever is due in no small part to the sensitivity of the antigens used. This, in turn, is dependent on the strains of organisms used and the method of preparation of the antigen. In this study an effort was made to select strains of *Escherichia typhosa* of high sensitivity and to prepare them in the most effective way.

O antigens were prepared according to the methods of Gilbert, Coleman, and Liviano,<sup>4</sup> and Mudd.<sup>17</sup> The turbidity of the final suspensions was adjusted to standard No. 3 of the MacFarland nephelometer. No difference was found in the sensitivity of the antigens prepared by either method. Since the Mudd method was simpler, it was used in preparing all other O antigens. Nine different O antigens were prepared.

Antigens prepared by pooling several different strains of organisms seemed to yield somewhat less satisfactory results than did single strains of highly

\*From the Bureau of Bacteriology of the Maryland State Department of Health, Baltimore.  
Received for publication June 27, 1938.

sensitive organisms. The pooled antigen showed only the degree of sensitivity associated with the weaker strains. The addition of the more active strains failed to compensate for the weaker ones. The importance of using a highly sensitive O antigen cannot be overstressed.

No difficulty was encountered in securing highly sensitive H antigens from actively motile strains of *Eberthella typhosa*. Several of these were compared, and all yielded similar titers.

H and O agglutination tests were incubated overnight at 52° C. with the level of the serum dilutions above the level of the water in the bath to create convection currents in the tubes. Titers were based on the highest dilution in which a definite (2+ or higher) agglutination reaction was obtained.

#### SELECTION OF SERA FOR STUDY

Over 3,000 sera were examined in this study. When there was sufficient material, the blood clots were cultured in Kracke-Teasley medium.<sup>18</sup> Preliminary microscopic agglutinations with living typhoid and paratyphoid B antigens were carried out in serum dilutions of 1:40 and 1:80, and those sera which showed definite agglutination (2+ or higher)\* after one hour at 37° C. in the 1:40 dilution were then titrated by the tube method for H and O agglutinins in dilutions from 1:20 to 1:5,120. Furthermore, only those sera which showed O titers of at least 1:80 were selected for study since it had been found that *Eberthella typhosa* is rarely isolated from any case with a lower concentration of O agglutinins. All cases reported as typhoid during the course of this study and on which agglutination tests were made, were found to have had an O agglutination titer of at least 1:80. Two hundred and eighty-eight sera were studied in this way. These were divided into three groups (1) 181 sera from reported cases of typhoid, (2) 69 sera from cases not reported as typhoid, although considerable O agglutinins were present, and (3) 38 sera from febrile nontyphoid cases. In addition, sera from a fourth group of 25 persons who had received antityphoid inoculations were studied.

Specimens were received for the most part in Keidel tubes. After removal of the serum for agglutination tests, the clot was cultured. Special blood cultures were also received from some cases and stool specimens from others.

Sera from 99 of 181 reported cases of typhoid had microscopict† as well as H and O titrations carried out in dilutions from 1:20 to 1:5,120. Titers determined by the microscopic agglutination test using a living antigen have been found to bear no constant relationship to either the H or O agglutination titers.

#### RESULTS OF STUDY

*Group I. Cases Reported as Typhoid.*—An O agglutination titer of 1:160 or greater was found in over 90 per cent of reported typhoid cases in spite of the fact that many specimens were taken early in the course of the disease.

\*4+ = practically all bacilli clumped; 3+ = approximately 75 per cent clumped; 2+ = 50 per cent; + = 25 per cent.

†The microscopic tests were made using standard size loopfuls of accurately diluted serum and antigen suspensions mixed on cover slips and examined microscopically.

H agglutinin titers were found to be much lower than O. Twenty one per cent (20.9) of the 181 reported typhoid cases had H titers less than 1:160 as compared to 88 per cent for O agglutination, whereas only 47.0 per cent had H titers of 1:640 or greater compared to 74.6 per cent with similar O titers (Table I).

In this group of 181 reported typhoid cases, there were 6 (3.3 per cent) which showed an O titer of 1:320 or higher but no H titer at all, and 13 (7.1 per cent) which showed an O titer of 1:320 or higher and an H titer not higher than 1:40. All except three gave microscopic titers comparable to the O titer. *Escherichia typhosa* was isolated from the blood of 61 of the 181 reported cases and from stool or urine specimens of 36 others.

TABLE I  
H AND O AGGLUTINATION TITERS IN 181 CASES REPORTED AS TYPHOID

Titer	Blood Positive <i>E. typhosa</i> 61 Sera		Stool Positive <i>E. typhosa</i> 36 Sera		<i>E. typhosa</i> Not Isolated 84 Sera		All Cases 181 Sera	
	O	H	O	H	O	H	O	H
	Per cent		Per cent		Per cent		Per cent	
1:80	11.5	18.0	8.3	22.2	7.1	21.4	8.8	20.4
1:160	6.6	21.3	5.6	19.9	8	20.2	7.2	19.3
1:320	6.7	14.7	2.8	16.6	14.3	10.7	9.4	13
1:640 or higher	75.1	46.0	83.3	47.7	70.1	47.7	74.6	47.0

**Group II Cases Not Reported as Typhoid**—Sera were examined from 69 cases with O titers of 1:80 or higher which were not reported as typhoid. Of these, 17 (24.6 per cent) gave O titers under 1:160, while the remaining 52, or 75.4 per cent, had titers of 1:160 or greater (Table II). It seems likely that a large number of these may have been unrecognized or unreported cases of typhoid in view of the fact that 71 per cent of the 69 had O titers of 1:320 or greater, while in a group of 38 nontyphoid cases (see Table III) and 25 persons given antityphoid inoculations (Table IV) only 2 (vaccinated) had O titers as high as 1:160. That some of these cases were undoubtedly typhoid is further supported by the isolation of *Escherichia typhosa* from several blood cultures. If these 52 cases were typhoid fever as the evidence indicates, it is possible that nearly a quarter (22.3 per cent) of the typhoid cases (181 + 52) were never reported as such.

TABLE II

H AND O AGGLUTINATION TITERS IN 69 SERA WITH O TITERS OF 1:80 OR GREATER NOT REPORTED CLINICALLY AS TYPHOID

	O	H
	Per cent	Per cent
Under 1:160	24.6	34.8
1:160	4.4	17.4
1:320	14.5	18.8
1:640 or higher	56.5	29.0

**Group III Febrile Nontyphoid Cases**—Sera were examined from 38 nontyphoid cases. On the basis of agglutination reactions, 10 were tularemia, 5 undulant fever, 4 spotted fever, and 3 infectious mononucleosis. In 16 in

stances in which no diagnosis was made by the physician, the laboratory tests were negative. The O agglutination titer for typhoid was 1:80 or less in all these cases previously (Table III).

TABLE III  
CLASSIFICATION AND O TITERS ON 38 FEBRILE NONTYPHOID CASES

DIAGNOSIS	CASES	O AGGLUTINATION
Tularemia	10	Partial 1:40
Undulant fever	5	Partial 1:80
Spotted fever	4	Partial 1:40
Infectious mononucleosis	3	Partial 1:80
Undiagnosed	16	Partial 1:80

*Group IV. Vaccinated Individuals.*—The sera of 25 individuals who had been given antityphoid inoculations were tested for their O agglutinin titers.\* Only 2 of the 25 sera had titers of 1:160. Nine had been inoculated within a year and the rest from one to three years (Table IV).

TABLE IV  
H AND O TITERS ON 25 PERSONS GIVEN ANTITYPHOID INOCULATIONS

HISTORY	PERSONS	O TITER
Inoculations within year	9	Highest 1:80
Inoculations one to two years previous	10	Highest 1:80
Inoculations two to three years previous	6	(2) 1:160; (4) 1:80

## DISCUSSION

The titer which is accepted as an indication of positive serologic reaction for typhoid fever should be (a) sufficiently high that it will seldom be found in nontyphoid cases, but (b) low enough that it will be of value in early diagnosis. If too high a titer is used, a large number of reactions will have to be reported as suspicious or indeterminate and other specimens obtained. While the examination of multiple specimens is highly desirable, it is not always possible for a physician to obtain these, and he may construe a suspicious report on a typical case as contraindicating the diagnosis of typhoid fever.

When highly agglutinable O antigens have been used, a titer of 1:160 has been found sufficiently high to constitute a positive serologic test for typhoid fever. This titer is found early enough (in the course of the disease) to be practical, and yet is rarely found, after six months, in persons who have been given antityphoid inoculations or who are suffering from infections other than typhoid. In our experience *Eberthella typhosa* has seldom been isolated from any case of typhoid when the O titer has been less than 1:80.† In the vast majority of cases of typhoid, an O titer of 1:160 or greater has been found with the first specimen submitted to the laboratory, and in practically all cases where *Eberthella typhosa* has been isolated from the blood stream, the O titer has been so high (1:640 or greater in 75 per cent of cases) that no reasonable question could be raised as to the diagnosis. In cases where the O titer was

\*The authors are indebted to Dr. H. E. Miller, of the 1392nd Company C.C.C. Camp D-2-Md., Goldsboro, Maryland, for blood from vaccinated persons.

†One or two isolations have been made subsequent to this study.

comparatively low on the first specimen submitted, a high titer was invariably obtained in subsequent specimens (O agglutinins have been found considerably earlier in the course of the disease than the H agglutinins). In a series of 38 febrile nontyphoid cases, all of the sera had agglutination titers below 1:160. Furthermore, sera from only 2 of 25 vaccinated individuals had titers as high as 1:160. Our results therefore are in agreement with those of Felix,<sup>7</sup> Smith,<sup>2</sup> Gardner,<sup>6</sup> and Lewin,<sup>4</sup> who suggested that a 1:200 titer was diagnostic, or, to be more exact, since our dilutions did not include 1:200, we feel that a titer of 1:160 may be considered diagnostic. The 1:500 titer of Dulaney and others<sup>12</sup> has been found to be too high with the antigens used in this study. Since a rise in titer seems to be the best indication of infection, we would agree with Bole<sup>10</sup> and others that it is best to carry out the complete titration each time rather than to use only one or two dilutions.

Sera from a number of cases of undiagnosed illness were found to have an O agglutination titer of 1:160 or higher. The agglutination reactions were so high in most cases as to leave little question of typhoid infection and in two cases, *Eberthella typhosa* was isolated from blood cultures. Although these had not been so reported, many of them probably were typhoid fever. It is therefore, possible that nearly a quarter of the typhoid cases considered in this study were never reported as such. These findings warrant a health department in promptly investigating all cases which show positive serologic tests for typhoid fever and in collecting blood and stool specimens from these cases regardless of whether or not they are clinically positive. This observation is in accord with that of Sellers<sup>20</sup> who estimated typhoid fever cases in Georgia for 1935 on the basis of deaths as 2,500 against only 1,013 reported.

There has been a tendency in recent years to disparage the microscopic Widal test with a living antigen. The use of dried blood is, of course, a crude and inaccurate method, though it has been of very great value. In our study of over 3,000 cases of possible typhoid fever, the microscopic agglutination test carried out with a sensitive, smooth, living antigen has been found to be almost as valuable from a diagnostic standpoint as the macroscopic test using the newer H and O antigens, provided the amount of serum available is sufficient for accurate dilutions and the technical worker has had experience in the reading of the microscopic test. Occasionally the O titer is considerably higher and hence of greater diagnostic value than the titer with the living antigen, although at times the reverse may be the case. H and O antigens can be carefully standardized, readings are less susceptible to individual error, difficulties in antigenic and cultural variations can be avoided, and the O antigen is agglutinated earlier in the course of the disease. In addition, the O antigen has been found of value in detecting carriers.

The routine use of an H antigen for typhoid has not been found of practical value. Its use is indicated in those cases in which a vaccinated individual is suspected of having typhoid. Cruickshank<sup>21</sup> has pointed out that H agglutinins have frequently been found to be absent in typhoid cases. While we have not observed this phenomenon to any extent, we have not found the routine use of H agglutination of value. The recent work of Damon<sup>22</sup> confirms

the work of others that H agglutination is not always specific. Also, the H agglutination may, at times, be partly or totally an anamnestic reaction.

Repeated blood specimens have been found to be of great value in cases with comparatively low initial titers or in cases in which there is some question as to diagnosis. We have failed, however, to obtain positive blood cultures in many typhoid cases which have almost invariably given high O agglutination titers with our sensitive antigens.

Nearly all of the 181 sera received from reported cases of typhoid in Maryland, including 61 sera from which positive blood clot cultures had been obtained, showed at least partial O agglutination in a dilution of 1:80. H agglutinins were present in a titer below 1:80 in 7.1 per cent of the cases and entirely absent in only 3.3 per cent of the cases.

#### SUMMARY

On the basis of this study, an O agglutination titer of 1:160 with an antigen of satisfactory sensitivity may be considered as a positive serologic test for typhoid, while a titer of 1:80 is suggestive and should be checked by further specimens.

Of approximately 3,000 sera examined during 1935 and 1936, 313 were selected on the basis of agglutination reactions with a sensitive living antigen and further studied for their H and O titers.

Sera were examined from 181 reported typhoid cases. In 61 of these, *Eberthella typhosa* was cultured from the blood; in 36 others, from stool specimens. All sera had at least a 2+ O agglutination in a dilution of 1:80. Ninety-one and two-tenths per cent had an O titer of 1:160 or greater, 84 per cent a titer of 1:320 or greater, and 74 per cent a titer of 1:640 or greater.

Of the 61 sera confirmed by positive blood clot cultures, 75.4 per cent showed an O titer of 1:640 or greater, 81.9 per cent an O titer of 1:320 or greater, and 88.6 per cent an O titer of 1:160 or greater.

The titers on sera from 38 febrile, nontyphoid patients and from 25 individuals who had received antityphoid inoculations, were less than 1:160, except in two of the vaccinated individuals.

In routine work, H titrations have not been found of value, except with sera from individuals known to have been vaccinated.

Even though reasonably reliable results can be obtained by experienced workers with the microscopic Widal test using satisfactory living antigens, there are so many practical advantages in the use of an O antigen suspension that it should be routinely employed.

The authors wish to acknowledge the valuable criticisms of this paper made by Elizabeth Petran and Ruth Myers of this department and by Thomas C. Grubb, formerly of this department.

#### REFERENCES

1. Felix, A.: Serologische Untersuchungen an Fleckfieberkranken aus der asiatischen Türkei, *Ztschr. f. Immunitätsforsch. u. exper. Therap.* 26: 602, 1917.
2. Smith, J.: The Serological Diagnosis of Typhoid and Paratyphoid Fevers, *J. Hyg.* 32: 143, 1932.
3. Lewin, W.: H and O Agglutination of *B. typhosus* in a Group of Suspected Typhoid Cases and in a Group of Unselected Individuals, *South African M. J.* 8: 731, 1934.

- 4 Gilbert, R, Coleman, M B, and Luzzano, A B A Study of the Granular and Floccular Types of Agglutination With *B typhosus*, *J LAB & CLIN MED* 19 225, 1934
- 5 Dulancey, A D, Wikle, W T, and Trigg R H and O Agglutinins as an Aid to the Diagnosis of Typhoid Fever, *Am J Pub Health* 22 1037 1932
- 6 Gardner, A D The Small Flaking, or O Agglutination of Permanent Standardized "O" Suspensions of *I typhosus* by the Serums of Normal, Inoculated and Infected Persons *J Hyg* 28 376, 1929
- 7 Felix, A The Qualitative Receptor Analysis in Its Application to Typhoid Fever, *J Immunol* 9 115, 1924
- 8 Wong D H, and Zin, S H Comparative Study of Various Typhoid O Antigens *Proc Soc Exper Biol & Med* 29 1149 1932
- 9 Foord, A G, and Lorch, A The Agglutinin Content of Blood Following Vaccination *Am J Clin Path* 3 333 1933
- 10 Wylie, J Experiences With Small Flaking or Granular Agglutination in Normal Inoculated and Enteric Fever Cases, *J Hyg*, 32 375 1932
- 11 Hergan, L S Qualitative or Quantitative Methods in the Serological Diagnosis of Enteric Infections<sup>2</sup> *J Hyg* 32 323, 1932
- 12 Dulancey, A, Wikle, W T, Stewart R L, Hatfield I D, Walker J K and Precher A B H and O Agglutination Following Typhoid Vaccination and in an Unselected Group of Individuals, *J Immunol* 24 229 1933
- 13 Gilbert, R, and Coleman M B Agglutination in the Diagnosis of Enteric Disease *Am J Pub Health* 23 693, 1933
- 14 Whitehead N T The Floccular and Granular Agglutinins in Typhoid Fever, *J Roy Army Med Corps* 55 81, 1910
- 15 Bertie C P and Elliott, T S Serological Diagnosis of Enteric in the Inoculated *J Hyg* 37 36, 1937
- 16 Gigholi, G Agglutinins Found in the Serum of Subjects Inoculated With Typhoid Paratyphoid Vaccine, *J Hyg* 33 387, 1933
- 17 Mudd, S Agglutinin Response to Typhoid Paratyphoid Vaccine, *J Immunol* 23 81, 1932
- 18 Kracke, R R, and Tersley, H E The Efficiency of Blood Cultures, *J Lab & Clin Med* 16 169, 1930
- 19 Bole, R Value of H and O Agglutination Technique in Routine Widal Reactions *J Lab & Clin Med* 20 638, 1935
- 20 Sellers T F Practical Procedures in the Laboratory Diagnosis of Typhoid and Clinically Related Fevers *Am J Pub Health* 27 159 1937
- 21 Cruickshank, J C Modern Methods in Agglutination, *Proc Roy Soc Med* 29 848, 1936
- 22 Damon, S R Agglutinins for Typhoid Group Bacilli in the Sera of Patients With Tuberculosis, *Am J Hyg* 26 40 1937



## OBSERVATIONS ON THE USE OF QUINIDINE SULFATE IN CHILDREN\*

CHARLES R. MESSELOFF, M.D., NEW YORK, N. Y.

THE action of quinidine sulfate on the human heart has been the subject of numerous investigations. These have established (1) that quinidine produces definite electrocardiographic changes;<sup>1-7</sup> (2) that it is rapidly eliminated from the cardiac structures;<sup>5-9</sup> and (3) that little cumulation of the drug takes place in the heart.<sup>5, 6</sup> Due, no doubt, to the fact that the more serious types of cardiac arrhythmias are infrequent during childhood, studies on the use of quinidine in man have been limited to adults. However, it was deemed desirable to know whether the observations relating to the action of the alkaloid on the human adult heart are applicable to that of the child. Accordingly, the effects of this drug, at various dosages, were studied on 12 children: 10 with regular sinus rhythm, 1 with frequent and persistent premature auricular contractions, and 1 with bundle branch block associated with congenital heart disease.

These observations revealed four significant points with reference to the use of quinidine during childhood: (1) Impairment of A-V conduction and of intraventricular conduction as well as decrease in the amplitude of the positive T-wave, phenomena noted in adult studies,<sup>4, 6, 7</sup> are also seen in children. (2) Quinidine may be safely administered to a child in doses proportionally very large for the age and the weight of the subject. (3) The principles of dosage and of cumulation governing the clinical use of the drug in children appear to be identical with those reported in adults. (4) A further point of interest is the fact that intraventricular conduction in the child with the bundle branch block was not unduly impaired by quinidine. The details of the observations made on the 12 children, together with a discussion of several features of special interest, form the subject of the present report.

### OBSERVATIONS

1. *Observations on Children With Regular Sinus Rhythm.*—Quinidine was administered to 10 children with rheumatic heart disease who were attending the Cardiac Clinic. Their ages ranged from 8 to 14 years. All had regular sinus rhythm and none presented signs of congestive heart failure. The initial dose in every case was 9 grains daily, and this was gradually increased until they were taking 45 grains daily in divided doses. Despite the large doses employed, extracardiac evidence of quinidine toxicity, gastrointestinal, visual, or auditory, did not appear in a single instance.

\*From the Cardiac Clinic of the Hospital for Joint Diseases.  
Received for publication, July 1, 1933.

The earliest and most constant electrocardiographic change was a decrease in the amplitude of the positive T wave in all leads. This occurred in every child in this group and appeared to be a very sensitive reaction to quinidine therapy. In some cases it was the sole evidence of quinidine effect on the heart, and appeared with doses as small as 9 grams daily in a few children.

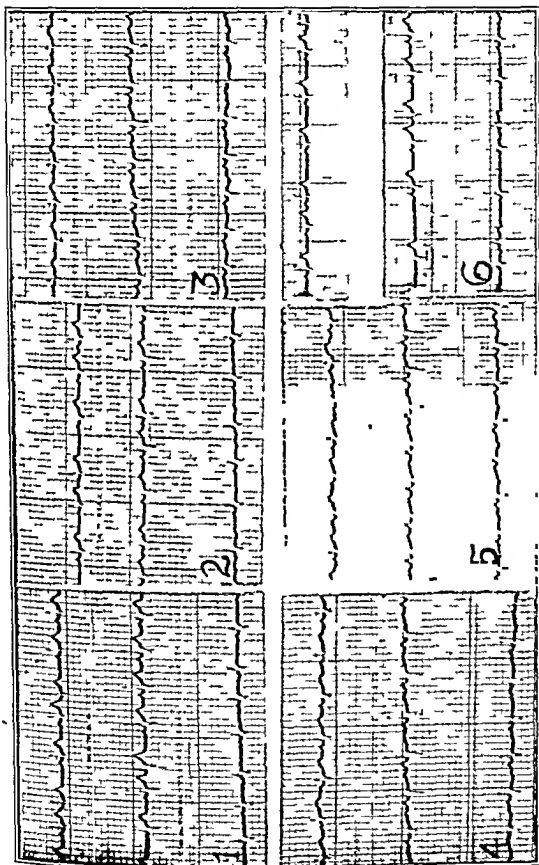


Fig 1.—Electrocardiograms of patient C.H. showing effect of quinidine: 1. Before quinidine; 2. after 15 grains daily for one week; decreased amplitude of T wave; 3. after 30 grains daily for one week; abolition of premature auricular contractions; prolonged QRS time; further depression of T wave; 4. after 30 grains daily for two weeks; maximum effects on QRS time and T wave; 5. after 30 grains daily for five weeks; persistence of effects noted after two weeks of medication; 6. after discontinuance of quinidine for two weeks; return of QRS time and T wave to normal values and reappearance of the premature auricular contractions.

Prolongation of the QRS time, ranging from 25 to 50 per cent of the control values, was present in 4 of the 10 cases. This increase of the QRS time appeared only when the level of dosage was stepped up to 30 grains. A-V conduction was found impaired in two instances, and then only during the period of dosage of 21 to 30 grains daily. In one case the control P-R interval

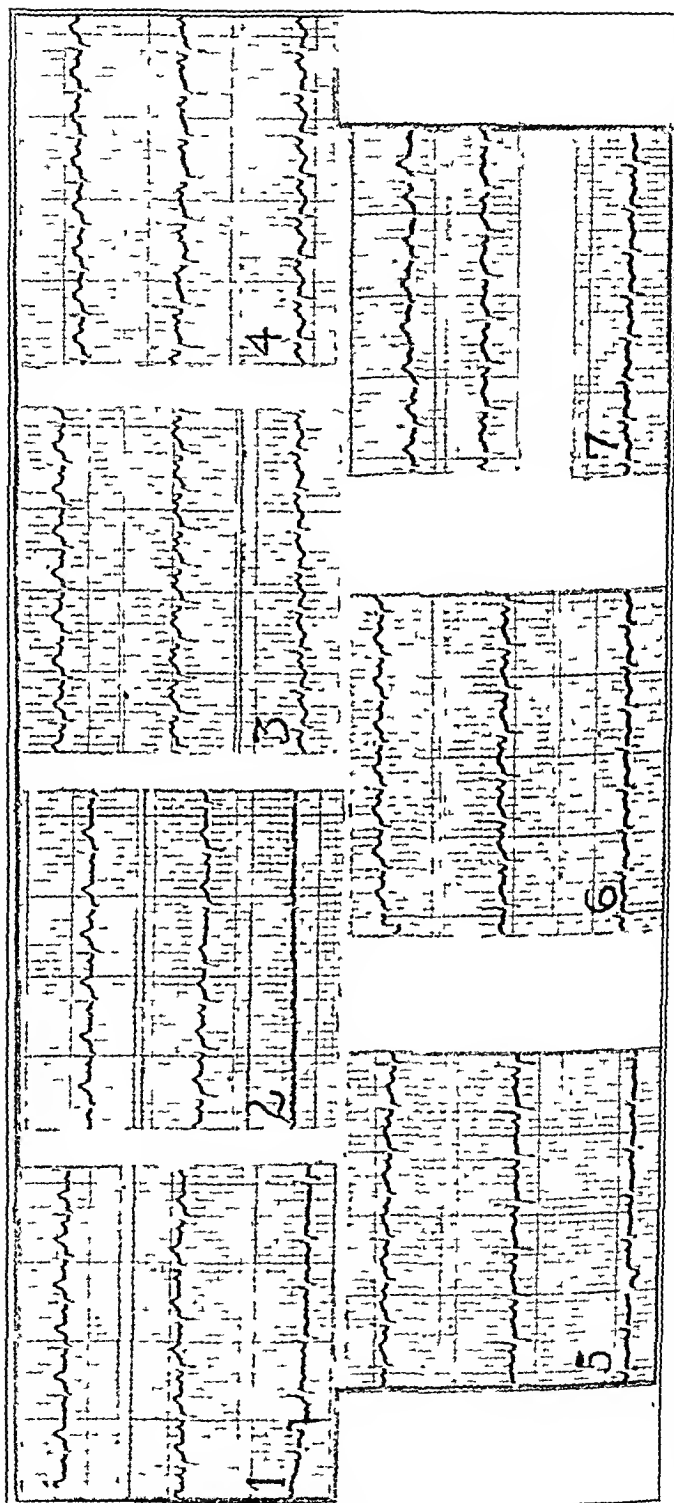


Fig. 2.—Electrocardiograms of patient C. H., showing the effect of a single 15-grain dose of quinidine. 1, Before quinidine; 2, thirty minutes after quinidine; 3, one hour after quinidine; 4, two hours after quinidine; 5, three hours after quinidine; 6, four hours after quinidine; 7, five hours after quinidine. The T-wave is still almost isoelectric, ventricular premature contraction. 6, four hours after quinidine, amplitude of T-wave returning to normal; 7, five hours after quinidine, further increase in the amplitude of the T-wave, ventricular premature contraction.

of 0.16 increased to 0.18 second, and in the other case the control P-R interval of 0.16 increased to 0.22 second.

2. *Observations on a Child With Premature Auricular Contractions.*—C. II., a boy 10 years old, was referred to the Cardiac Clinic for a study of a cardiac arrhythmia discovered in the course of a routine physical examination. The final diagnosis established in the clinic was regular sinus rhythm with premature auricular contractions, Class E (possible heart disease).<sup>4</sup>

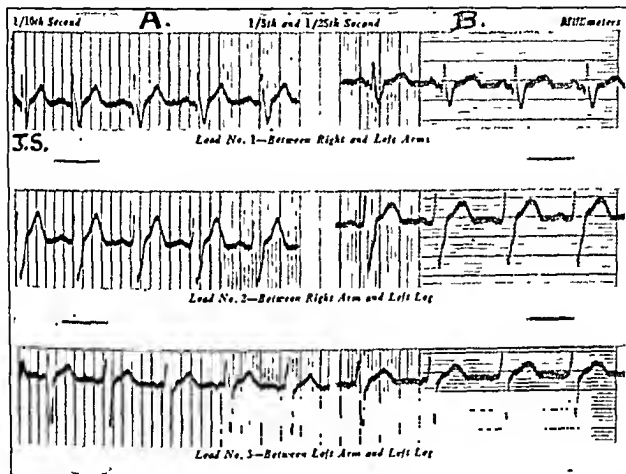


Fig. 3—Electrocardiograms of patient J. S. A, Before quinidine, shows P-R interval of 0.16 second and QRS time of 0.12 second. B, after 30 grains quinidine daily for four days, shows P-R interval of 0.18 second and QRS time of 0.16 second.

The effect of the drug on the premature contractions is shown in Figs. 1 and 2. These show that when quinidine was administered in divided daily doses, it was necessary to increase the level of daily dosage to 30 grains in order to abolish the premature contractions. On the other hand, one single dose of 15 grains sufficed to abolish the ectopic beats within one hour and to prevent their reappearance for at least five hours (Fig 2). The clinical results of quinidine administration in this child differed in no appreciable way from those obtained in adults with a similar arrhythmia.<sup>10 12</sup>

Electrocardiographically, there was evidence of depression of both A-V and of intraventricular conduction during the period of dosage of 30 grains daily, the P-R interval having increased 29 per cent and the QRS time 25 per cent above the original values. A lowering of the amplitude of the positive T-wave appeared after one week of 15 grains daily, while the maximum decrease of about 50 per cent below the control reading appeared only at the level of daily dosage of 30 grains.

\*Following the nomenclature adopted by the New York Heart Association.

The maximum effects of the single dose of 15 grains consisted of a 27 per cent decrease in the amplitude of the positive T-wave and a 10 per cent increase in the QRS time (Fig. 2). These changes appeared within two hours, which is about the same time required in adults for a similar dose to produce its greatest effect.<sup>2, 6</sup> The electrocardiographic changes seen in this child, both during the period of divided daily doses and after the single large dose, were essentially the same as those observed in adults.<sup>6</sup> Attention should, however, be called to the fact that others<sup>13</sup> have failed to find impairment of either A-V or of intraventricular conduction after the administration of as much as 60 grains of quinidine daily. An explanation of this difference cannot be offered at this time.

3. *Observations on a Child With Bundle Branch Block.*—J. S., a boy 6 years old, was referred to the Cardiac Clinic with a diagnosis of congenital heart disease. The diagnosis after complete examination was heart disease of congenital origin, interventricular septal defect, enlarged heart, normal sinus rhythm, bundle branch block, and no diminution of the functional capacity (Class 1).\*

In order to observe the effect of quinidine on a heart with a bundle branch conduction defect, the drug was given in gradually increasing doses, beginning with 6 grains daily. It was found that doses up to 9 grains daily failed to produce any change in the electrocardiogram, while doses of 15 and 20 grains daily caused a prolongation of the QRS time by about 17 per cent. A dose of 30 grains daily for four days further increased the QRS time up to 33 per cent above its original value (Fig. 3). No significant changes were seen in the P-R interval, the T-wave, or the sinus rate. These findings are in complete accord with those of Gold and his co-workers<sup>6</sup> who observed no unusual susceptibility of an adult with bundle branch block, given large doses of quinidine, to the depression of intraventricular conduction.

#### COMMENT

The average daily dose of quinidine in adults is from 15 to 30 grains, although 60 grains and more have been administered daily for long periods of time without untoward effect.<sup>6, 7, 14</sup> The average adult daily dose was exceeded in every one of the 10 children with regular sinus rhythm, 45 grains of the drug having been taken daily for two weeks. None of them showed symptoms of cinchonism or developed serious conduction disturbances or arrhythmias. These observations suggest that the full average adult dose may be safely given to children rather than the fraction of this dose based either on the age or on the weight of the subject. Nevertheless, since great individual variation in sensitivity to quinidine is known to exist, it is advisable to check the degree or the presence of conduction impairment by frequent tracings when large doses are employed, as was done in the present study.

No appreciable cumulation of the drug in the cardiac structures was in evidence at any time. Thus, the daily administration of 30 grains daily for five weeks to the child with extrasystoles produced no greater changes in his electrocardiogram at the end of this period than after one week of medication.

\*Following the nomenclature adopted by the New York Heart Association.

However, it should be emphasized that inasmuch as observations were made only at weekly intervals, it is not surprising that cumulation was not noted in any of the cases studied, since cumulation on a fixed daily dose occurs only during the first few days of medication<sup>6</sup>

It should be further noted that a study of Figs 1 and 2 shows very clearly that it is the size of the individual dose and not the total period of dosage that determines the magnitude of the effects of quinidine. Thus, one 15 gram dose was more effective in abolishing the premature auricular contractions and was productive of greater electrocardiographic changes than a similar amount given in 5 gram doses three times daily for one week. Moreover, the clinical and electrocardiographic changes produced by the single large dose were practically identical with those noted after the prolonged administration of 30 grams daily but divided into three 10 gram doses.

The findings in the children studied, both as they relate to dosage and to cumulation of quinidine, are essentially the same as those reported by Gold and his co-workers<sup>6</sup> in their study on the use of the alkaloid in ambulatory adult cardiac patients.

#### SUMMARY AND CONCLUSIONS

1 Observations were made on 12 children relative to the action of quinidine sulfate upon their hearts. The results, clinical and electrocardiographic, were compared with published findings in adults.

2 The electrocardiographic changes produced by the drug in children were found to be identical with those noted in adults. The clinical results in a child with frequent premature auricular contractions differed in no appreciable manner from those seen in an adult with a similar arrhythmia. Published observations that quinidine produced no unusual depression of intraventricular conduction in an adult with bundle branch block were confirmed in a child with a similar conduction impairment.

3 Children tolerate large doses of quinidine as judged by the age and weight standards. The usual adult daily dose of 15 to 30 grams may be safely given to children. It is advised, however, to control the large doses by frequent electrocardiograms.

4 The principles underlying dosage and cumulation of quinidine established for adults apply equally to children.

#### REFERENCES

- 1 Lewis T, Drury, A N, Ihescu, C C, and Wedd, A M. Observations Relating to the Action of Quinidine Upon the Dog's Heart, With Special Reference to Its Action on Clinical Fibrillation of the Auricles, *Heart* 9 55 1921
- 2 Lewis T, Drury, A N, Wedd, A M, and Ihescu, C C. Observations Upon the Action of Certain Drugs Upon Fibrillation of the Auricles, *Heart* 9 208, 1921
- 3 Korns H M. An Experimental and Clinical Study of Quinidine Sulphate, *Arch Int Med* 31 15, 1923
- 4 Wilson, F N, and Wishardt, S W. The Effects Produced by the Intravenous Injection of Quinidine and Other Drugs Upon the Mechanism of the Heart Beat, *Tr A Am Physicians* 41 55 1926
- 5 Gold, H, and Modell, W. The Action of Quinidine on the Heart in the Normal Unanesthetized Dog, *J Pharmacol & Exper Therap* 46 357, 1932
- 6 Gold H, Otto, H L, and Satchwell H. The Use of Quinidine in Ambulatory Patients for the Prevention of Paroxysms of Auricular Flutter and Fibrillation, With Especial Reference to Dosage and the Effects on Intraventricular Conduction, *Am Heart J* 9 219, 1933

7. Viko, L. E., Marvin, H. M., and White, P. D.: A Clinical Report on the Use of Quinidine Sulphate, *Arch. Int. Med.* 31: 345, 1923.
8. Weiss, S., and Hatcher, R. A.: Studies on Quinidine, *J. Pharmacol. & Exper. Therap.* 30: 335, 1927.
9. Gordon, B., Matton, M., and Levine, S. A.: The Mechanism of Death From Quinidine and a Method of Resuscitation: An Experimental Study, *J. Clin. Investigation* 1: 497, 1925.
10. Barrier, C. W.: The Use of Quinidine in the Treatment of Ectopic Rhythm, *J. A. M. A.* 89: 742, 1927.
11. Smith, F. M.: Quinidine in the Treatment of the Cardiac Irregularities, *J. A. M. A.* 78: 877, 1922.
12. Otto, H. L., and Gold, H.: Persistent Premature Contractions, *Arch. Int. Med.* 38: 186, 1926.
13. Maher, C. C., Sullivan, C. P., and Seheribel, C. P.: The Effect Upon the Electrocardiogram of Patients With Regular Sinus Mechanism of Quinidine Sulphate, *Am. J. M. Sc.* 187: 23, 1934.
14. Levine, S. A., and Fulton, M. N.: The Effect of Quinidine Sulphate on Ventricular Tachycardia, *J. A. M. A.* 92: 1162, 1929.

1750 GRAND CONCOURSE

---

## THE EFFECT OF BANANAS ON LAXATION\*

---

PHILIP L. HARRIS, PH.D., CHARLESTON, S. C., AND GEORGE L. POLAND, B.S.,  
NEW YORK, N. Y.

---

**P**RELIMINARY to a clinical investigation concerning the laxative effect of various fresh fruits, a short exploratory study was carried out on rats. Normal male albino rats, 90 to 100 days of age and representing eight different litters, were used. The banana was chosen for study as a typical fresh fruit which is readily available, widely used, and of constant composition. Furthermore, several conflicting opinions which have been expressed regarding the laxative value of bananas, should be reconciled if possible. Bergeim and co-workers,<sup>1</sup> Eddy,<sup>2</sup> Russell,<sup>3</sup> and von Meysenbug,<sup>4</sup> have proved to their satisfaction that bananas have a definite laxative value. Other investigators, Thompson,<sup>5</sup> Northmann,<sup>6</sup> Pease and Rose,<sup>7</sup> are equally convinced that this fruit has no influence on bowel movement. However, the American Medical Association, Committee on Foods,<sup>8</sup> has allowed the advertising claim that "Ripe bananas . . . are an aid against constipation," thereby recognizing that bananas have at least a regulating effect upon laxation.

The present experiment was divided into three periods of fourteen days each. During the first period the animals were fed the breeding colony diet consisting of  $\frac{2}{3}$  whole wheat,  $\frac{1}{3}$  whole milk powder, and 1 per cent sodium chloride. This same ration plus 20 gm. of banana pulp from fruit not completely ripe (yellow peel with green tip) was fed during the second period. The third period was identical with the second, except that the banana pulp was from fully ripe bananas (yellow peel with brown flecks). One week was allowed between periods II and III, during which the rats received only the basal diet so that no effect of the second period would be carried over to the third.

Of the six criteria for determining degree of laxation proposed by Cowgill,<sup>9</sup> only three were considered practical for use in this study, viz., (1) amount of

---

\*From the Medical College of Charleston and the Research Laboratory, United Fruit Co., New York.

intestinal content evacuated per day, (2) water content of the feces, and (3) laxation rate or number of defecations per day. Consequently, each day at the same hour feces from each individual animal were collected, counted, and weighed. At the end of each week, during the experimental periods, the total collected feces from each rat were dried, total solids were determined, and water content was calculated. The results are shown in Table I.

TABLE I

SHOWING THE LAXATIVE EFFECT OF BANANAS WHEN FED AT DIFFERENT STAGES OF RIPENESS

	FEEDING PERIOD*				
	I	II		III	
	AVERAGE OF RESULTS	AVERAGE OF RESULTS	PER CENT INCREASE OVER PERIOD I	AVERAGE OF RESULTS	PER CENT INCREASE OVER PERIOD I
Laxation rate (number fecal units per day)	15.9	20.9	31	23.9	50
Weight of feces (mg per day)	1230.0	1760.0	43	1940.0	58
Dry weight of feces (gm per week)	11.5	12.9	12	14.2	23
Water content (per cent)	33.2	47.6	43	47.7	44
Increase in body weight (gm)	18.0	24.0	33	38.0	111

\*Feeding period I—Animals received the complete colony diet for fourteen days.

Feeding period II—Animals received the colony diet plus twenty gm of banana (yellow skin with green tip) daily for fourteen days.

Feeding period III—Animals received colony diet plus 20 gm of banana (yellow peel specked with brown) daily for fourteen days.

From a consideration of these data it appears that bananas, either partially or fully ripe, do exert a laxative effect. This effect in rats is probably not due to the crude fiber of the bananas, since the 20 gm of banana pulp contained less crude fiber than the equivalent amount of basal diet for which it was substituted. Neither does it seem probable that the vitamin B<sub>1</sub> or mineral content of the banana supplements was the factor which produced increased laxation, since the basal diet was liberally supplied with both of these essentials.

A consideration of the relative effects of partially ripe and completely ripe bananas upon the laxation criteria indicated that the laxation principle is present in larger quantities in completely ripe than in partially ripe bananas. The differences in chemical composition of bananas at these two different stages of ripeness are small. Slightly less starch and free tannin, and slightly more sugar and pectin occur in the completely ripe fruit. The small relative amounts of starch and sugar which the banana supplement introduced into the diet eliminate these as causative factors. The free tannin content decreases and the pectin content increases as the banana ripens because the tannins are slowly bound in an insoluble, supposedly inert, "vegetable tannate," while the pectins are slowly freed from an insoluble, inert protopectin. Consequently, the presence of more bound tannin and more free pectin in the fully ripe banana than in the partially ripe fruit should be considered in explaining the superior laxative potency of the fully ripe fruit.

The accelerated gain in body weight during the second and third periods is an anomaly difficult to explain, since the food energy intake increased only slightly during these periods.

Occasional reports<sup>10</sup> of subjective impressions that the laxation benefits from bananas decreased after the first few days of exceptional success in a few human



subjects, were investigated and tested in these experiments. The laxation rates, water content of the feces, and daily amount defecated were averaged for the first and second half of each period and compared in Table II.

TABLE II

A COMPARISON BETWEEN THE RESULTS OF THE FIRST AND SECOND HALF OF EACH EXPERIMENTAL PERIOD

	PERIOD I (CONTROL)			PERIOD II (PARTIALLY RIPE BANANAS)			PERIOD III (FULLY RIPE BANANAS)		
	FIRST HALF	SEC- OND HALF	PER CENT CHANGE	FIRST HALF	SEC- OND HALF	PER CENT CHANGE	FIRST HALF	SEC- OND HALF	PER CENT CHANGE
Laxation rate (number of fecal units per day)	15.3	16.6	+ 8	21.4	20.5	-4	26.2	21.6	-18
Weight of feces (mg.)	1140.0	1320.0	+16	1790.0	1730.0	-3	1960.0	1920.0	- 2
Water content of feces (per cent)	33.0	33.4	+ 1	46.9	48.3	+3	46.7	48.7	+ 4

The subjective impression, that the ability of bananas to increase daily defecations decreased during the course of the experimental periods, was not substantiated in these experiments on rats. In only one instance, the laxation rate during period III, was the performance during the second half of the period markedly decreased over that of the first half.

## CONCLUSION

The results of this experiment indicate three facts that may be of value in planning future clinical studies concerning the effect of bananas upon laxation. These are (1) that fully ripe bananas (yellow peels flecked with brown), are more laxative than partially ripe bananas (yellow peels with green tips); (2) that free pectin and bound tannin may prove to be the principal laxation factors of the banana and consequently the intake of these compounds should be determined in both the control and experimental periods; and (3) that there is apparently a sustained increase in laxative effect due to banana feeding in the rat which may also occur in human subjects. Nevertheless, a clinical study should be extended enough so that a relatively great preliminary stimulating effect upon laxation, if present, would not be given undue weight in the final averages.

## REFERENCES

1. Bergeim, O., Hanszen, A., and Arnold, L.: Influence of Fruit Ingestion Before Meals Upon the Bacterial Flora of the Stomach and Small Intestines, *Am. J. Digest. Dis. & Nutrition* 3: 97, 1936.
2. Eddy, W. H.: Nutritive Value of the Banana, Bureau of Publication, Teachers College, Columbia University, New York, 1933.
3. Russell, H. K.: The Banana, Life and Health, page 8, Oct., 1935.
4. von Meysenbug, L.: Feeding During the First Year, With Reference to Some of Its Problems, *Arch. Pediat.* 44: 215, 1927.
5. Thompson, W. G.: Practical Dietetics, ed. 4, New York, 1909, D. Appleton-Century Co.
6. Northmann, H.: *Die Banane*, *Medizet* 9: 392, 1926.
7. Pease, M. C., and Rose, A. R.: The Banana as a Food for Children, *Am. J. Dis. Child.* 14: 379, 1917.
8. American Medical Association, Committee on Foods, *J. A. M. A.* 97: 1890, 1931.
9. Cowgill, G. R., and Anderson, W. E.: Laxative Effect of Wheat Bran in Healthy Men, *J. A. M. A.* 98: 1866, 1932.
10. Eddy, W. H.: Personal communications.

## RAGWEED HAY FEVER THE EFFECT OF DOSAGE IN THE TREATMENT OF 314 CASES\*

HAROLD D. DUNOY, M.D., LOUIS LAVIN, M.D., AND HARRY MARKOW, M.D.,  
BROOKLYN, N. Y.

SPECIFIC pollen therapy is generally accepted as the modern method of treatment of hay fever. The procedure is fairly well standardized and the results of treatment, as tabulated in recent years are generally accepted to indicate that which might be expected from an average case receiving average treatment with any of the various extracts now obtainable. From the various reports of results of specific pollen therapy, the present method of treatment is likely to yield from 80 to 85 per cent satisfactory results, with 5 to 10 per cent of all cases uninfluenced by therapy.

A study of the literature, however, reveals a variety of factors, which influence the results of such treatment. Among these factors are mentioned (a) the type of treatment perennial versus preseasonal, (b) dosage high or low, (c) route of treatment subcutaneous or intracutaneous, (d) the type of pollen extract used. A special study has therefore been made of a fairly large group of hay fever patients to determine the relative merits of these factors in the treatment.

Rackemann,<sup>1</sup> reviewing the results of the treatment of ragweed hay fever, concluded that larger doses, in the main, produced better end results, that excessive dosage was the cause of failure in a small percentage of the cases, and that the results of several years of treatment were little influenced by other factors, such as pollen counts, etc. Cohen, Reicher, and Breitbart<sup>2</sup> reported better results in the treatment of hay fever with large doses, but this procedure required a greater number of injections for the season. They believed that the main cause of failure was the inability to produce a tolerance to sufficiently large dosage of pollen extract required for protection against the concentration of pollen in the air during the ragweed season. Rammer<sup>3</sup> concluded, on the basis of a large series of cases, that good results depended upon the administration of large amounts of pollen extract before the onset of the season. Markow and Spain<sup>4</sup> emphasized the importance of high levels of dosage. Egermann<sup>5</sup> used small dosage in his treatment, but concluded that one of the causes of failure was inadequate dosage. Brown<sup>7</sup> was very emphatic in maintaining that the results of treatment of seasonal hay fever are directly proportional to the size of the maximum dose obtained before the onset of the season. He used a top dose of 60,000 to 100,000 Noon pollen units (i.e. of a 6 per cent to 10 per cent

\*From the Department of Medicine Division of Applied Immunology Beth El Hospital Brooklyn

Received for publication July 12 1938

pollen extract). Similarly, Waldbott,<sup>5</sup> and Waldbott and Ascher<sup>8</sup> found that a maximum amount of relief was obtained with maximum doses, usually of about 15,000 Noon units. On the other hand, Clarke<sup>9</sup> obtained optimal results with a low top dosage (800 Cooke units or 0.015 mg. of total nitrogen) with no ragweed injections during the season. The general consensus of opinion seems to be that the perennial method of treatment gives about 10 per cent better results than the preseasonal method,<sup>10-12</sup> with only an occasional dissenting opinion.<sup>13</sup>

In this study, a review of a large group of hay fever cases, involving five years of treatment, has been made. The factors involved in this study were chosen mainly for their influence on results of treatment and consisted of the route of injection, the method of treatment (preseasonal and perennial), and the various top dose levels.

TABLE I  
COMPOSITE CHART  
1933 to 1937 Inclusive

		NUMBER OF CASES	PER CENT PERFECT RESULTS	PER CENT GOOD RESULTS	PER CENT POOR RESULTS
ALL CASES		314	5.2	78.9	16.0
Method of treatment	Preseasonal	239	2.9	77.9	19.2
	Perennial	75	12.0	82.7	5.3
Type of extract	Defatted ragweed	108	3.7	78.7	17.6
	Whole ragweed	206	5.8	79.2	15.0
Top dose for season	0.01 mg. N per c.c.	35	0	62.9	37.1
	0.05 mg. N per c.c.	105	2.9	76.1	21.7
	0.1 mg. N per c.c.	113	3.5	86.8	9.7
	Over 0.1 mg. N per c.c.	61	14.8	78.6	6.6
Method of injection	Intracutaneous	210	5.9	79.8	14.3
	Subcutaneous	104	3.8	77.0	19.2
Sex	Males	186	3.2	82.9	13.9
	Females	128	7.8	73.4	18.8
Degree of skin sensitiveness	Class A	5	0	80.0	20.0
	Class B	104	1.9	80.8	17.3
	Class C	205	6.8	78.1	15.1

The patients received daily symptom cards, upon which they recorded the degree of hay fever symptoms for each day of the season. These were checked each week upon their appearance at the clinic, and corrected whenever necessary. All patients were tested by the intracutaneous method and classified according to Vander Veer.\* Each patient was tested and retested at the beginning of the treatment, and when necessary, further testing was done to determine the degree of sensitiveness. Two types of extracts were employed: the regular ragweed extract, made according to the method of Coca, and a whole ragweed extract similarly made, except that it was not defatted. Injections were given weekly, and an attempt was made to reach the top dose for each class by the onset of the season. This was impossible in many cases because of a late start and irregularity of attendance. However, this study includes all cases having had sufficient treatment for the purpose of tabulation. Arbitrarily they have

\*Described by Vander Veer, Cooke, and Spain in the Am. J. M. Sc, 174: 101, 1927.

been divided into four groups. The first group included those cases that failed to receive doses higher than 0.01 mg. of total nitrogen,\* the second group received the top dose of 0.05, the third group received the top dose of 0.1, and the fourth group included all cases that exceeded the top dose of 0.1.

The common denominators in this group of patients were (1) they were all ragweed hay fever cases, both clinically and on skin tests, (2) they were all treated with ragweed pollen extract either defatted or nondefatted.

The variable factors that were considered were as follows: (1) sex, (2) method of treatment (preseasonal or perennial), (3) dosage, (4) type of pollen extract used, (5) route of treatment (subcutaneous or intracutaneous) and (6) degree of sensitiveness of patient as revealed by intracutaneous testing.

#### RESULTS AND COMMENTS

It will be noted that the results obtained for all cases approximate the reports of most observers, i.e., about 84 per cent of satisfactory results. It appears that skin sensitiveness, sex, type of extract, and route of administration do not seem to influence results. However, important and significant differences do appear as regards dosage and method of treatment (preseasonal or perennial). Here it will be noted that as the top dose is increased, the percentage of poor results is markedly decreased, so that the group receiving the top dose of 0.1 or more have a much lower percentage of poor results than the general average for the year, or the composite average for the five years. The cases receiving perennial treatment approximated the results obtained in the high dose group. It must be remembered, however, that the perennial cases were in the main in the high dose group. It is our opinion, therefore, that the apparent superiority of the perennially treated over the preseasonally treated cases is primarily one of high dosage.

This study supports the generally held view that better results are due to high dosage in the treatment of ragweed hay fever. From our findings, one can hardly explain the excellent results occasionally reported by others with low dosage therapy. Certainly, the reported failures in treatment with the perennial method are easily understood when associated with what seems to us to be extremely low dosage for this type of treatment. It would seem that the perennial method of treatment is the method of choice in those patients who can tolerate large doses at long intervals. Unfortunately, however, this method is not applicable to those individuals who, in the course of treatment, develop an intolerance to pollen extract. We have thought it wise to omit from consideration in this paper the results of a detailed investigation of the causes of failure in our small group of unsatisfactory results, including such factors as secondary sensitiveness to foods and inhalants, secondary paranasal sinus infections, poorly timed constitutional reactions, and other causes generally noted in such reports.

#### CONCLUSIONS

1. Results of specific therapy in late hay fever bear a definite relationship to the dosage of extract administered. In this study doses of ragweed extract of 0.1 or over yielded better results than the composite average for the whole group.

\*Hereafter in referring to dosage of pollen extract it will be understood that the figures refer to milligrams of total nitrogen.

2. An important factor in the apparent superiority of perennial over the preseasonal method of treatment is the high dosage employed in this form of treatment. Low dosage in the perennial form of treatment yields unsatisfactory results.

3. All other factors considered, namely, type of extract, sex of patient, degree of sensitiveness, and the route of injection, are shown to have no appreciable effect upon therapeutic results in hay fever.

#### REFERENCES

1. Rackemann, F. M.: The Optimal Dosage in the Treatment of Hay Fever, *J. Immunol.* 11: 91, 1926.
2. Cohen, M. B., Reicher, J., and Breitbart, J.: The Causes of Failure in the Treatment of Hay Fever, *Ohio State M. J.* 24: 372, 1928.
3. Ramirez, M.: Statistical Report on the Treatment of Hay Fever, *Am. J. M. Sc.* 176: 856, 1928.
4. Markow, H., and Spain, W. C.: Ragweed Hay Fever; Its Diagnosis and Treatment, *J. Allergy* 1: 414, 1930.
5. Waldbott, G. L.: The Causes of Failure of the Pollen Treatment for Hay Fever, *J. Michigan M. Soc.* 29: 567, 1930.
6. Eyermann, C. H.: The Treatment of Hay Fever, *J. Missouri M. A.* 28: 191, 1931.
7. Brown, G. T.: Maximum Dosage in Pollen Therapy, *J. Allergy* 3: 180, 1932.
8. Waldbott, G. L., and Ascher, M. S.: The Optimal Doses in Pollen Therapy, *J. Allergy* 8: 457, 1937.
9. Clarke, J. A., and Leopold, J. C.: Prophylactic Treatment for Ragweed Hay Fever, *J. Allergy* 8: 560, 1937.
10. Unger, L.: Perennial vs. Preseasonal Treatment of Hay Fever, *J. Allergy* 3: 545, 1932.
11. Vander Veer, A.: The Relative Merits of Seasonal and Perennial Treatment of Hay Fever, *J. Allergy* 7: 578, 1936.
12. Spain, W. C., and Fuchs, A.: A Clinical Study of the Advantages of the Perennial Treatment of Hay Fever, *South M. J.* 30: 1199, 1937.
13. Peshkin, A.: Critique of the Perennial Treatment of Pollen Allergy, *J. Allergy* 7: 477, 1936.

---

## THE EFFECTS OF TOBACCO SMOKING ON HEALTH\*

---

### A STUDY OF 2031 MEDICAL RECORDS

---

JAMES J. SHORT, M.D., F.A.C.P., HARRY J. JOHNSON, M.D., F.A.C.P., AND  
HAROLD A. LEX, JR., A.B., NEW YORK, N. Y.

---

DEBATES and controversies regarding the alleged harm to the human system from the use of tobacco still continue. The literature on the subject, extensive and often conflicting, varies in type from statistical studies on mortality, such as recently reported by Pearl,<sup>1</sup> to single experiments on individuals. Prejudice and emotion have so often entered into the problem that the question presents many complexities to the investigator who earnestly desires unbiased information on the relationship of tobacco consumption to the public health. A discussion of this subject, together with a review of the literature and a bibliography, has recently been published by Thienes and associates.<sup>2</sup>

\*From Medical Examinations by the Life Extension Examiners, New York.  
Received for publication, July 12, 1938.

In an attempt to learn what, if any, symptoms are produced in comparatively healthy ambulatory individuals by the use of tobacco, an unselected group of insurance policy holders were asked to fill out a questionnaire at the time of their periodic health examination. The questionnaire was designed to elicit their practices with reference to the use of tobacco, but contained no reference to physical signs or symptoms. This latter information was obtained independently from the physical examination and medical history which were recorded separately. Thus, by separating the time and method of obtaining the two kinds of information, it was hoped to avoid suggestion and bias.

Of 2,031 cases studied, 1,292, or 63.7 per cent, habitually used tobacco, 496, or 24.4 per cent, were nonusers, 104, or 5.1 per cent, used it only occasionally, and 139, or 6.8 per cent, were former users who had discontinued the practice.\* No sex differentiation was made in this study. Chart 1 shows the trend in amount consumed by habitual users, though it must be admitted that the figures are only approximate. Weights were computed so that equivalent amounts might be included, regardless of method of use, whether through the smoking of cigarettes, cigars, or pipes. It will be noted that there was a tendency for increased consumption for each five year period of use up to thirty years, when a sharp decline was noted.

Distribution of habitual smokers, according to duration of use, was as follows:

YEARS	NUMBER	PER CENT
1-4	118	9.1
5-9	197	14.9
10-14	243	18.8
15-19	247	19.1
20-24	191	14.8
25-29	121	9.4
30-34	87	6.7
35-39	40	3.1
40-44	33	2.6
45-49	12	0.9
50-54	7	0.5

Subjective symptoms and physical signs of smokers and nonsmokers are listed in Table I. Symptoms relating to the respiratory, circulatory, gastro-intestinal, and nervous systems are definitely increased among smokers. These increases range from 50 per cent for palpitation of the heart to 300 per cent for cough. The increased tendency to dyspnea on exertion noted by the smokers gives point to the taboo on tobacco by many athletic directors, especially by the trainers of pugilists.

The relationship of tobacco smoking to gastric disturbances, especially to hyperacidity, is a frequent clinical observation. For this reason sufferers from gastric or duodenal ulcers are usually induced to refrain from tobacco. In this study, "heartburn" was increased by 100 per cent, and other digestive symptoms from 62 to 112 per cent.

That the two groups listed in Table I were fairly comparable is indicated by the average ages, which were nearly identical. There was no significant variation between the two groups in weight, pulse rate, or blood pressure. Tremors appeared to be diminished in the smoking group. The writers<sup>3</sup> have

\*This does not represent the average practice of the population at large.

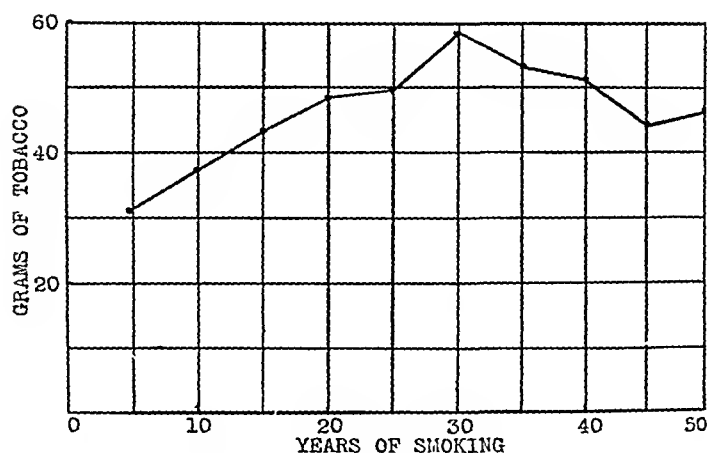


Chart 1.—Average amounts of tobacco consumed for each five-year period of use

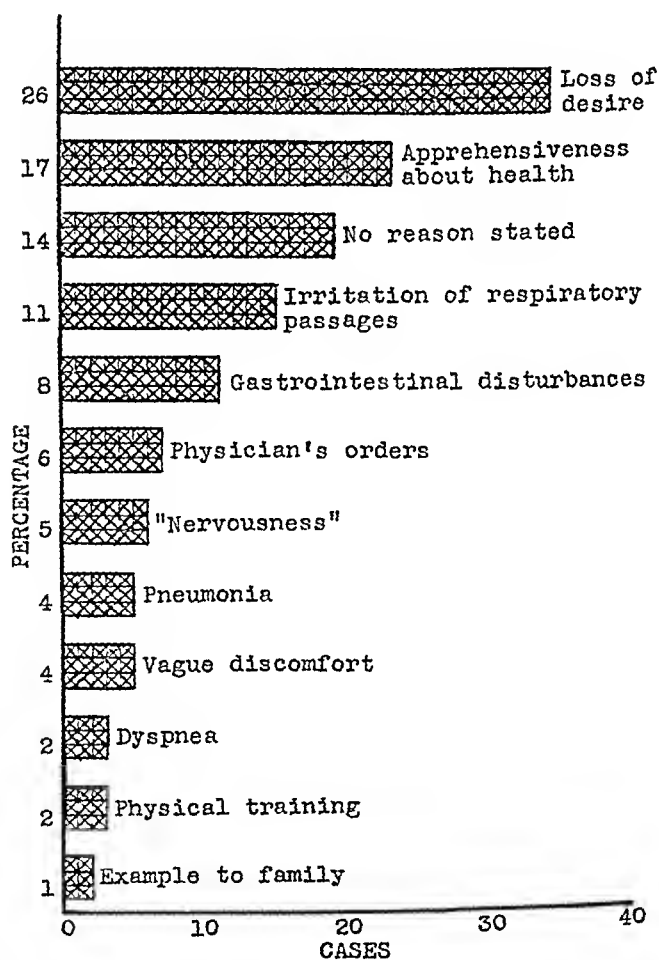


Chart 2.—Reasons given for discontinuing the use of tobacco.

TABLE I  
SYMPTOMS, FINDINGS, AND DIFFERENCES FOR SMOKING AND NONSMOKING GROUPS

SYMPTOMS AND FINDINGS	NONSMOKERS		SMOKERS		PERCENTAGE INCREASE OR DECREASE
	NUMBER	PERCENT	NUMBER	PERCENT	
<i>Symptoms</i>					
Respiratory system					
Irritation of nose and throat	12	24	82	64	+167
Cough	8	16	83	64	+300
Frequent colds	54	109	223	180	+15
Circulatory system					
Palpitation	14	28	54	42	+50
Precordial pain or "stress"	28	56	125	97	+73
Dyspnea on exertion	24	48	148	115	+140
Gastrointestinal system					
"Heartburn"	3	6	15	12	+100
Excessive gas	62	127	266	206	+12
Miscellaneous	28	77	211	163	+112
Nervous system					
"Nervousness"	19	38	86	67	+76
<i>Findings</i>					
Tremors	57	115	129	100	13
Premature heart beats	2	4	8	6	+50
Systolic heart murmurs	41	82	161	105	+27
Average age	68		72		+1
Average weight	157		154		+0.2
Average pulse	78.5		78.2		0.3
Average blood pressure	121/76		121/78		0/+3

noted, however, that there is a tendency for tremors to be increased if habitual users are too long deprived of tobacco, also that blood pressures tend to vary considerably during the actual process of smoking.

Reasons given for the discontinuance of tobacco by 139 individuals are graphed in Chart 2. Seventy-eight, or 56 per cent in this group gave up smoking for reasons of health.

#### COMMENT

The complaints recorded are of especial significance because they were entirely spontaneous, written by the examinee on a history form and not elicited by questioning. It should be emphasized that both groups were treated exactly alike and the findings therefore cannot be biased. We believe that the findings in this study indicate a trend in accordance with the recent report of Pearl who found mortality markedly increased among heavy smokers. It is hoped that further studies will demonstrate more definitely the manner in which tobacco exerts its influence and the chief hazards attached to its usage.

#### REFERENCES

- 1 Pearl, R. Tobacco Smoking and Longevity, Science 87: 216, 1938.
- 2 Thienes, C. H., and Butt, E. M. Assisted by Bartlett, J. C., Behrendt, A., Fieldin, F., Messenger, T. T., and Rollins, E. Chronic Circulatory Effects of Tobacco and Nicotine, Am J M Sc 195: 522, 1938.
- 3 Short, J. J., and Johnson, H. J. Unpublished data.



# A DIRECT COMPARISON OF THE REACTIONS OF THE HUMAN SYSTEM TO TOBACCO SMOKE AND ADRENALIN\*

JAMES J. SHORT, M.D., F.A.C.P., AND HARRY J. JOHNSON, M.D., F.A.C.P.,  
NEW YORK, N. Y.

FOLLOWING the observations that the smoking of cigarettes produced a lowering of peripheral skin temperatures<sup>1-3</sup> and elevation of blood sugar,<sup>4</sup> it was considered of interest to make a direct comparison of the effects of adrenalin injection with those of tobacco smoking. Our procedure is here described.

## PROCEDURE

Five normal habitual smokers in the postabsorptive state, after preliminary control observations, were studied for skin and oral temperature, blood pressure, and pulse rate changes, and for blood sugar and cholesterol fluctuations for one and one-half hours while cigarettes were being smoked. The skin temperature observations were made on a continuously recording apparatus by means of a thermocouple attached to the dorsum of the terminal phalanx of the finger. The blood pressures and pulse rates were taken at five-minute intervals; the oral temperatures and blood specimens for cholesterol and sugar at fifteen-minute intervals.

One week later the same subjects were treated in exactly the same way, except that, instead of the smoking of cigarettes, 1 c.c. of 1:1000 adrenalin solution was injected subcutaneously, following the control observations. Because of the rather large volume of individual observations and the tendency for wide fluctuations to occur, the results have been compiled as composite rather than as individual curves.

A comparison of the findings is expressed graphically in Charts 1-5.

## FINDINGS

Chart 1 shows a remarkable parallelism in the pulse rates during tobacco smoking and after the injection of adrenalin solution. There was a decided increase which reached a peak at the end of about one-half hour and gradually diminished thereafter. The blood pressures generally showed a similar parallelism, but with striking fluctuations which were not altogether ironed out even in the composites. The most striking part of the blood pressure study was the lowering of the diastolic pressure after adrenalin,<sup>4</sup> an effect which would hardly be expected on theoretical grounds, since adrenalin produces a peripheral vasoconstriction. A compensatory mechanism probably accounts for this phenomenon. The diastolic pressure after cigarette smoking remained more constant at about the normal average values.

\*From the Research Department of the Life Extension Institute, New York.  
Received for publication, July 12, 1938.

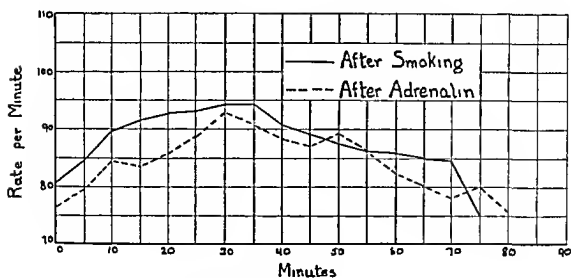


Chart 1.—Composite pulse rates after smoking and after adrenalin injection

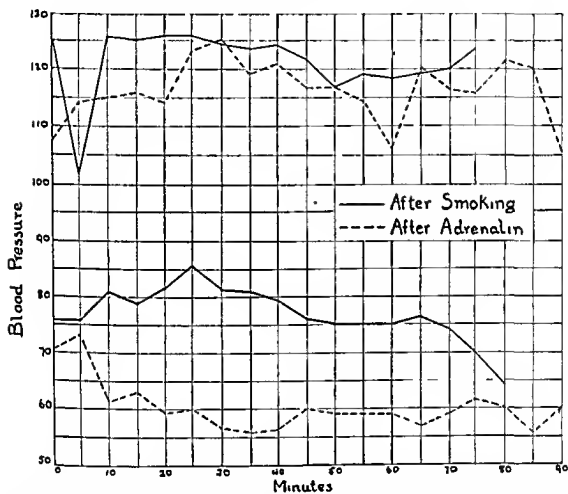


Chart 2.—Composite blood pressures after smoking and after adrenalin injection

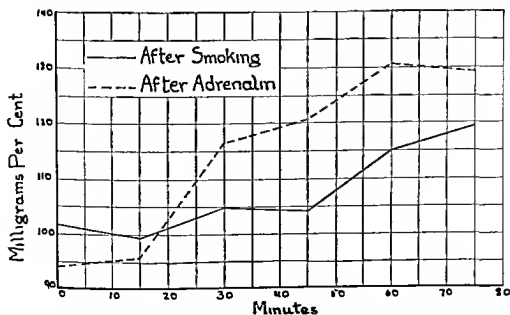


Chart 3.—Composite blood sugar curves after smoking and after adrenalin injection.

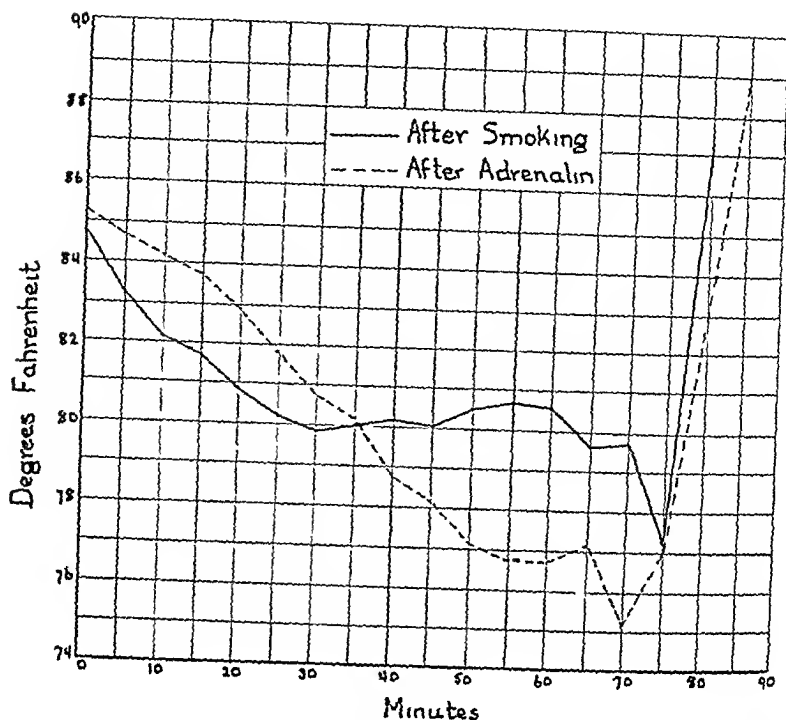


Chart 4.—Composite skin temperatures after smoking and after adrenalin injection.

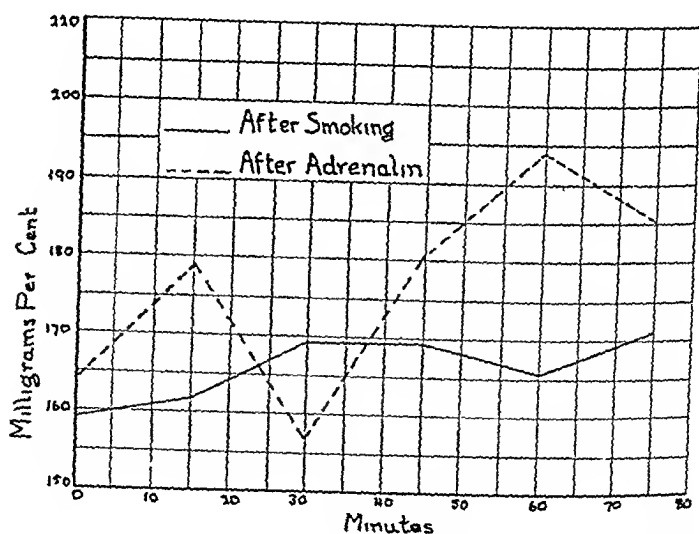


Chart 5.—Composite total blood cholesters after smoking and after adrenalin injection.

Confirming the observations of Haggard and Greenberg,<sup>5</sup> blood sugar values were seen to increase at fifteen-minute intervals from normal to an average upper value of 119 mg. during the seventy-five minutes of smoking. The results after adrenalin injection were similar but more pronounced. The composite curves of the skin temperatures during the two experiments were decidedly similar. During the smoking period the temperatures dropped from 84.6° F. to a low

point of  $77.4^{\circ}\text{F}$ . After adrenalin, the temperatures diminished from  $85.3^{\circ}\text{F}$  to a low point of  $75.2^{\circ}\text{F}$ . After a period of about one and one quarter hours, the temperatures during both experiments showed an abrupt increase to  $87.4^{\circ}\text{F}$  and  $89^{\circ}\text{F}$ , respectively.

Total blood cholesterol were also studied in these experiments. It was noted that there was a slight tendency to an increase during tobacco smoking and a more marked increase after adrenalin. The adrenalin experiment, however, produced marked fluctuations.

#### COMMENT

Sollman<sup>6</sup> states that blood pressure may be raised by nicotine, even in the absence of the vasomotor center, but not after the suprarenals have been excised. He attributes the blood pressure elevation to an increased output of epinephrine. Stewart and Rogoff reported an increased output of epinephrine after nicotine administration, followed by a marked and enduring decrease. Rogoff attributes this to a stimulation of the adrenal medulla through the sympathetic nervous system.

In the foregoing experiments, we believe that the parallelism between the effects of injected adrenalin and smoking of tobacco is most significant. That the results were not altogether identical was to be expected because of the impossibility of adjusting the dose of adrenalin to correspond to the increment added to the blood by nicotine stimulation. Then too, absorption of injected adrenalin is an unpredictable and variable factor.

#### CONCLUSION

Our observations confirm the view that the characteristic effects upon pulse, blood pressure, peripheral skin temperature, and blood sugar of tobacco smoking can be explained by an increased output of epinephrine. This most probably is the result of a stimulating effect of nicotine upon the sympathetico-adrenal system.

#### REFERENCES

- 1 Madoch, W. G., and Collier, F. A. Peripheral Vasoconstriction by Tobacco Demonstrated by Skin Temperature Changes, *Proc Soc Exper Biol & Med* 29: 487, 1932.
- 2 Short, J. J., and Johnson, H. J. Effect of Smoking on Skin Temperature, *J Lab & Clin Med* 19: 962, 1934.
- 3 Wright, I. S., and Moffat, D. Effects of Tobacco on Peripheral Vascular System, Further Study, *J A M A* 103: 318, 1934.
- 4 Elliot, A. H., and Nuzum, F. R. Cardiovascular Response to the Subcutaneous Injection of Epinephrin and Pituitrin in Essential Hypertension, *Am J M Sc* 189: 215, 1935.
- 5 Haggard, H. W., and Greenberg, L. A. The Effects of Cigarette Smoking on Blood Sugar, *Science* 79: 165, 1934.
- 6 Sollman, T. *Manual of Pharmacology and Its Application to Therapeutics and Toxicology*, Philadelphia, 1932, W. B. Saunders Co.
- 7 Rogoff, J. M., and Stewart, G. N. Quoted in Sollman, T. *Opus cit*.

The authors wish to acknowledge the courtesy of Dr. Irving Sherwood Wright in offering the use of the temperature recording apparatus of the Vascular Clinic of the New York Post-Graduate Hospital.

## HISTOLOGY OF DEMODEX FOLLICULORUM\*

EUGENE MAIER, PH.D., VENICE, FLA.

IN THE course of studies endeavoring to ascertain more efficient chemicals for the treatment of Demodex and Sarcoptic infection in man and animals, we obtained histologic sections of *Demodex folliculorum* which we are here presenting.

The mites exhibit a great variety of habits. They live principally on fluid nutriment which is obtained from living plants or animals or from decaying organic matter. The parasitic mites are of great interest on account of the wide variety of their habitats. Many like the ticks are external parasites of animals, feeding on the blood of their hosts; some like Sarcoptidae and Demodicidae, burrow in the skin of their hosts and cause severe itching and disease. Many species attack birds and feed on the scales and feathers, or even invade the lungs, air sacs, and hollow bones. Of still greater importance has been the discovery that many blood-sucking mites may serve as intermediate hosts of various pathogenic organisms of man and animals.

*The Demodicoidea.*—The Demodicoidea, the hair follicle mites, are a highly aberrant group of mites. They are parasitic in the hair follicles of mammals. They are elongate, the legs reduced to mere stumps, the abdomen vermiform, and the mouth parts modified, minute and fitted for piercing. The super-family contains but a single family, the Demodicidae and the one genus Demodex. The hair follicle mites of different animals are extremely difficult, if not impossible, to differentiate as distinct species.

*Demodex folliculorum* Simon is the hair follicle mite of man. It is abundant in some countries, but it is said to be rare in North Africa. It lies deep down in the hair follicles and the sebaceous glands. The entire life cycle is passed on the host, so that the infection gradually spreads.

*Demodex canis* Leydig, with which we are dealing here, attacks dogs and is cosmopolitan in distribution. It causes the follicular or red mange of the dog. The disease is serious. *D. cati* Megnin, the cat, *D. bovis* Stiles, cattle, *D. equi* Daillet, horses, and other species are formed on different mammals. Several workers have recorded *D. canis* parasitizing man. It would, however, appear quite probable that species commonly occurring in our domestic animals may attack man.<sup>1</sup>

*Characteristics of Demodex folliculorum.*—These wormlike mites attain a length of about 0.3 mm. and a width of 0.045 mm. They have lanceolate lyrate

\*From the Florida Medical Center, Venice, Fla.  
Received for publication, July 12, 1938.



Fig 1—This section shows a whole lesion with the hair shaft in the center. The most prominent feature is the circular wall around the lesion consisting of a rather heavy infiltration of round cells from without. The hair shaft in the center is cut tangentially and is in the process of autolysis. On top of the shaft a parasite is seen cut sagittally and below the shaft are two more parasites cut the same way. In the surrounding tissue adjacent to the shaft and within the wall of the lesion many parasites are seen most of them being cut transversely.



Fig 2—For details of the parasites this section is a high power enlargement of a small center part of Fig 1. Three parasites (a b c) are showing structural details especially a having eight pairs of stumpy legs and the capitulum showing clearly.

or horseshoe-shaped head, with shearlike masticatory apparatus, mobile proboscis, and triple-jointed maxillary antennae, an elongated cone-shaped abdomen, and eight triple-jointed stumpy feet attached to the thorax. The eggs are spindle-shaped. The larvae, when emerging from the egg, have the same general form as the fully equipped mites but have only six feet

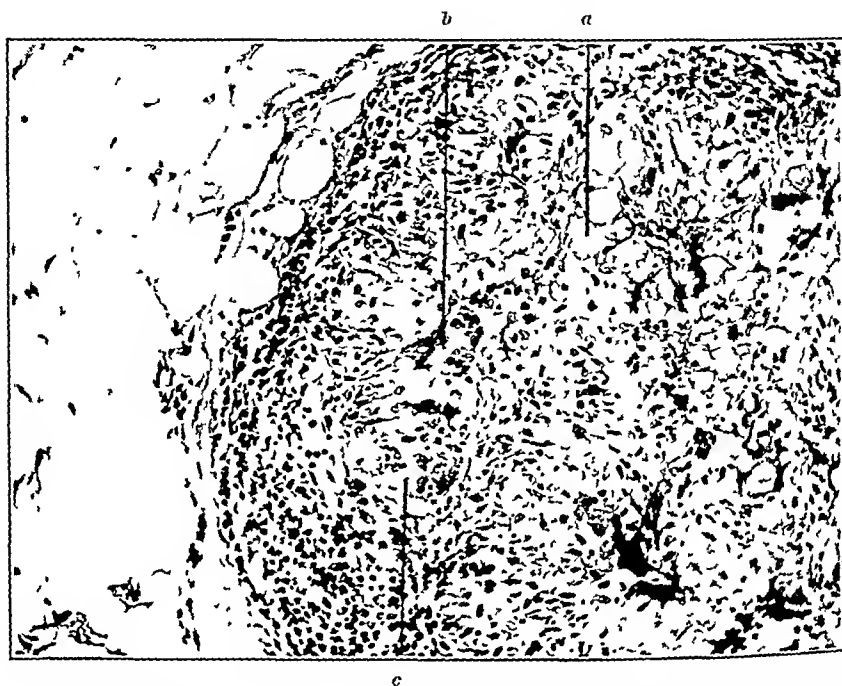


Fig 3—This section shows the isolation of the lesion by a heavy wall of connective tissue, infiltrated densely by round cells. Besides these pathologic changes this picture shows the formation of multinucleated giant cells (a, b, c) and the engulfing of a parasite (a) probably in its larval stage, i.e., active phagocytosis

These parasites are found in the hair follicles and sebaceous glands, their head toward the bottom of the sac and the caudal end toward the external opening. Their number may be large in a sebaceous gland, even as many as 150 having been found there. They generally average from 30 to 50. The accompanying photomicrographs confirm this statement. The larvae and eggs, often in great numbers, lie beside the parasite. The irritation of the parasite causes the follicles and sebaceous glands to become dilated, and the subsequent atrophy of the hair papillae causes falling out and permanent loss of hair.

#### SUMMARY

The histologic sections of *Demodex folliculorum* in the epidermis of the dog are described, and the appearance of the parasites in situ is shown in the photomicrographs.

#### REFERENCE

- 1 Todd, James C., and Sanford, Arthur H.: Clinical Diagnosis by Laboratory Methods. Philadelphia, 1927, W. B. Saunders Co

## THE RELATION BETWEEN SKIN SENSITIVITY, LIVER FUNCTION, LEUCOPENIC INDEX, AND TOXIC EFFECTS FROM CINCHOPHEN\*

WILLIAM B. RAWLS, M.D., F.A.C.P., BENJAMIN J. GELSKIN, M.D.,  
ANTHONY A. RESSA, M.D., AND ABRAHAM S. GORDON, M.D., F.A.C.P.  
New York, N. Y.

FOLLOWING our original observation<sup>1</sup> that amidopyrine produced agranulocytosis in certain patients, an extensive series<sup>2</sup> of patients receiving this drug was studied carefully. The reproducibility of the agranulocytosis, which was confined to 1 per cent of the patients suggested that it was caused by hypersensitivity to the drug, but skin sensitivity tests with aqueous solutions gave essentially negative results. Hirschfall<sup>3</sup> demonstrated that, while aqueous solutions of formaldehyde gave negative skin tests, a combination of blood serum with formaldehyde gave positive reactions. This suggested an explanation for our failure to obtain positive skin tests with aqueous solutions of amidopyrine. Damashek,<sup>4</sup> following the principle of Hirschfall and working with patients who had had agranulocytosis and in whom an etiologic relationship had been established, found that, while aqueous solutions of amidopyrine did not give positive skin tests or produce constitutional symptoms, as little as  $\frac{1}{6}$  gr. of amidopyrine mixed with blood serum and injected intradermally gave positive reactions and precipitated an attack of the disease in certain cases.

In view of our own experience,<sup>1, 2</sup> this was regarded as conclusive evidence that the agranulocytosis in these patients was due to drug hypersensitivity. It suggested that, since the toxic symptoms from amidopyrine were probably due to allergic phenomena, toxic symptoms from other drugs might also be due to allergy. Damashek<sup>4</sup> did not make intradermal tests on a large number of other patients and confirm the tests by oral administration of amidopyrine. It was considered advisable, therefore, to make further study along these lines, using a large series of patients and a drug which is more frequently toxic than pyrimidon, viz., phenylethionine acid (cinchophen), to determine (1) the relation between skin sensitivity, liver dysfunction, and the development of toxic symptoms, and (2) whether skin tests were of value for eliminating patients who are likely to develop toxicity from cinchophen. The leucopenic index was determined in 20 cases.

### MATERIALS AND METHODS

The patients were selected without discrimination on admission to the clinic. Skin sensitization tests and liver function tests were made on all patients before cinchophen was administered. Great care was taken to eliminate experimental

\*From the Arthritis Clinic of the New York Polyclinic Medical School and Hospital, New York.  
Received for publication July 15 1938.



TABLE I

RELATION BETWEEN INTRADERMAL SENSITIVITY, LIVER DYSFUNCTION, AND CINCHOPHEN TOXICITY

PATIENT	INTRADERMAL SENSITIVITY DILUTIONS			LIVER DYS-FUNCTION	TOXIC SYMPTOMS AFTER TAKING CINCHOPHEN							OTHER SYMPTOMS	
	I	II	III		NAU-SEA	URTI-CARIA	ICTER-US TINGE	RASH	ITCH	DIZZI-NESS	NERV-OUS-NESS		
Patients With Toxic Symptoms After Taking Cinchophen													
F. F.	0	++	++	+	+					+	+	Depressed	
O. K.	0	+	++	+	+					+	+		
M. R.	+	++	+	+	+					+			
C. O.	++	++	++	+	+	+							
J. G.	+	++	+++	+	+							Insomnia	
S. M.	++	++	0	+	+		+	+					
R. C.	+	++	+++	+	+					+	+		
F. B.	+	+	+	+	+					+	+		
F. P.	+	++	+	+	+							Vomiting	
C. G.	0	++	+	+		+							
H. S.	+	+	+	+				+	+				
M. D.	++	++	++	+						+	+		
E. D.	++	++	++	+						+	+	Irritable	
S. E.	+	++	+++	+					+		+		
S. C.	+	++	+++	+				+	+		+		
F. W.	+	++	+++	+						+	+		
M. M.	0	++	+++	+						+	+	Restlessness	
T. W.	0	++	++	+				+	+		+		
A. S.	0	0	0	+									Diarrhea*
A. N.	0	+	+	+				+	+				
C. R.	++	++	++	0	+								
M. S.	+	++	+	0	+				+	+			
P. S.	+	++	+++	0	+			+					
A. P.	++	+	+	0		+							
M. H.	0	+	+++	0	+					+			
F. B.	0	0	0	0		+					+		
N. D.	0	0	0	0					+				
M. G.	0	0	0	0					+	+			
M. M.	0	+	0	0			+		+				
J. T.	0	0	+	0		+							
Patients Having No Toxic Symptoms After Taking Cinchophen													
W. P.	0	+++	++	+									
S. C.	++	+	+	+									
C. S.	0	++	++	+									
C. C.	+	++	+++	0									
V. L.	+	++	+++	0									
L. O.	+	+	+	0									
E. T.	0	0	0	+									
J. B.	0	+	+	+									
J. P.	0	+	+	+									
M. M.	0	+	+	+									
M. R.	0	0	0	0									
E. W.	0	0	0	0									
L. B.	0	+	+	0									
M. C.	0	0	0	0									
T. R.	0	0	0	0									
A. G.	0	0	0	0									
R. L.	0	0	0	0									
T. M.	0	0	0	0									
C. T.	0	0	0	0									
O. K.	0	+	+	0									

\*Severe.

error, which is apt to occur in this type of work, and any suspicious findings were checked. Cinchophen given steadily was given for four weeks, provided toxicity did not develop.

A 5 per cent solution of sodium cinchophen was combined with blood serum as follows: 10 cc, 20 cc and 30 cc quantities of the cinchophen solution were mixed with 50 cc of blood serum, labeled dilutions No. I, II, and III, respectively, and kept in the refrigerator four days. As a control, 50 cc of blood serum mixed with 30 cc of normal saline also was placed in the refrigerator four days. The injections consisted of 0.05 cc intradermally on the forearm. Readings were taken in thirty minutes because the reactions reached a maximum by this time. Only those reacting 2+ or more with any of the dilutions, or 1+ with all dilutions, were considered positive. An occasional patient who gave a positive reaction with the control serum was not included in the positive group.

The azotubin S, hippuric acid and bilirubin excretion tests were used to determine liver function since we showed that they were the most reliable in chronic arthritis. A positive result with one or more of these tests was considered evidence of liver dysfunction.

The technique and interpretation of the leucopenic index were described by Vaughan.<sup>6</sup> The mean fasting white blood cell count was determined. One tablet (gr. viii) of cinchophen was crushed and given with a small amount of water to promote rapid absorption. Blood counts were taken after fifteen, thirty, forty-five, sixty, and ninety minutes and again after twenty-four hours.

The following symptoms were considered evidence of cinchophen intoxication: urticaria, scarlatiniform rash, angioneurotic edema, nausea and vomiting, jaundice, distention with abdominal pain, and loss of appetite, nervousness associated with depression, hematuria, and diarrhea.

*Analysis of 50 Cases*—Of the 50 patients studied, 30 (60 per cent) developed signs of toxicity attributed to cinchophen. Twenty-three (77 per cent) of this group gave positive skin reactions to cinchophen.

Twenty-nine (58 per cent) of the entire group gave positive skin reactions, and 23 of these (79 per cent) developed toxic symptoms. Seven (33 per cent) of the 21 patients with negative skin tests developed toxic symptoms.

In 6 patients, all with positive skin tests, the skin test dose alone produced toxic symptoms. Five of them also developed toxic symptoms after oral administration.

*Relation Between Liver Function, Skin Tests, and Cinchophen Toxicity*—Liver function tests were made on all 50 patients before cinchophen was administered. Fifteen (71 per cent) of those with negative skin reactions had normal liver function tests while 73 per cent of patients with positive skin tests had liver dysfunction. Sixty-seven per cent of the patients who developed toxicity showed evidence of liver dysfunction as compared with only 35 per cent of those who did not develop toxic symptoms. Of the 30 who developed toxicity, 60 per cent gave both positive skin and liver function tests, while 50 per cent of the 20 who did not develop toxicity gave both negative skin and liver function tests. The relationships confirm the opinion of previous investigators that liver damage may predispose to toxicity from cinchophen.

*Leucopenic Index.*—Vaughan<sup>6</sup> reported that, in certain patients with food allergy, there was a drop in the total leucocyte count after the ingestion of food to which they were sensitive. The leucopenic index, which has been used as a test for sensitivity to different agents, was done on 20 patients who had previously shown toxic symptoms after taking einchophen, and 9 (45 per cent) of them were positive. The leucopenic index, therefore, did not compare favorably with the skin test, which gave positive reactions in 77 per cent of the patients who developed toxicity.

#### DISCUSSION

One hesitates to propose a diagnostic procedure based on skin tests alone because they are prone to considerable error. Tests were repeated on the first 25 patients to determine the reproducibility of the tests and similar results were obtained in 21 (84 per cent). Frequent rechecking of other patients also gave similar results. However, the results with each dilution were not always constant. The intensity of the skin reactions was not proportional to the amount of drug injected. In some instances the reaction from dilution No. I, containing the least amount of einchophen, was more intense than those from dilutions No. II and III. In other instances, the reactions varied according to the concentration of einchophen-serum mixture. These findings suggest that the results can be used qualitatively but not quantitatively, and that the experimental error can be minimized by using different amounts of test substance and by rechecking doubtful results.

There did not appear to be any relationship between the intensity of the skin reactions, the interval before toxicity developed, and the severity of the toxic symptoms.

#### CONCLUSIONS

1. Seventy-nine per cent of the patients who showed positive intradermal tests with the einchophen-serum mixture developed toxic symptoms after einchophen was administered by mouth, while only 33 per cent of the patients giving negative tests developed toxic symptoms.

2. Seventy-one per cent of the patients with negative skin reactions had normal liver function tests, while 73 per cent of those with positive skin tests had liver dysfunction.

3. Liver dysfunction was present before treatment in 67 per cent of patients who developed toxic symptoms after taking einchophen by mouth, as compared with 35 per cent in those who did not develop toxic symptoms.

4. The leucopenic index did not prove to be as valuable as the intradermal tests for determining the likelihood of patients developing toxicity from einchophen.

5. The results of this work suggest that intradermal tests with drug-serum mixtures may be of value in determining drug toxicity and should receive further study.

#### REFERENCES

1. Rawls, W. B.: Neutropenia Developing During Amidopyrin Medication, *Am. J. M. Sc.* 187: 837, 1934.
2. Rawls, W. B.: The Effect of Amidopyrin Upon the Red, White and Polymorphonuclear Blood Cells of a Series of 100 Patients, *Am. J. M. Sc.* 192: 175, 1936.

- 3 Horsfall, F L, Jr Formoldehyde and Serum Proteins Their Immunological Characteristics, *J Immunol* 27 553, 1934
- 4 Dameshek, W and Colmes, A The Effect of Drugs in the Production of Agranulocytosis With Particular Reference to Amidopyrin Hypersensitivity, *J Clin Investigation* 15 85, 1936
- 5 Raulo W B, Weiss, S, and Collins, V L Liver Infection in Rheumatoid (Chronic Infectious) Arthritis, *Ann Int Med* (In Press)
- 6 Vaughn, W T Further Studies on the Leucopenic Index in Food Allergy, *J Allergy* 6 1, 1934

The authors wish to acknowledge the Director of the Calco the methods used in the charge by the Calco Co

lightness to Dr David A Bryce Medical many valuable suggestions in reference to used in this study was furnished without the courtesy of Dr Bryce

The authors also wish to express their thanks to Mr George H Chapman for his valuable suggestions during the preparation of this paper and to Miss Santa Tell who performed the technical studies

## COMPARISON OF INTRADERMAL TESTS WITH AGGLUTINABILITY AND CERTAIN IN VITRO TESTS OF STREPTOCOCCI, STAPHYLOCOCCI, MICROCOCCUS CATARRHALIS AND COLON BACILLI ISOLATED FROM PERSONS SUSPECTED OF HAVING CHRONIC INFECTION\*

GEORGE H CHAPMAN AND CONRAD BERLINS M D NEW YORK, N Y

INTRADERMAL tests have been used widely not only for the differentiation of "focal infection" bacteria but also in the selection of bacteria for the preparation of autogenous vaccines. The literature is too voluminous to be reviewed here. The following papers, however, have a special bearing on the subject.

Steinberg<sup>1</sup> presented an excellent critique of the limitation of skin tests in allergy.

Steinberg and Wiltsie<sup>2</sup> obtained reactions with *B. coli* toxic filtrate in all 60 normal children and all 40 normal adults. These results are similar to those obtained by workers in other allergens. For example, Gion and Herman<sup>3</sup> obtained 55.5 per cent positive results with common allergens in a group of 150 normal individuals.

Inconclusive results have been reported also in persons with different diseases. Steinberg and Wiltsie<sup>2</sup> found that the skin reaction to *B. coli* was not related to the presence of infection. Four of 11 patients with pyelitis did not react to *B. coli* toxic filtrate, and 5 of 11 did not react to *B. coli* vaccine. They concluded that "under the conditions of these experiments the skin reaction for the determination of the presence of colon bacillus infection is of uncertain value." Solis Cohen<sup>4</sup> concluded that "There probably is no relationship between hypersensitiveness in the host to the exogenous and endogenous toxins of a given organism and the pathogenicity of such organism for that host." His conclusion that intracutaneous tests are unreliable for selecting bacteria for vaccines seems to have been shared by most recent

\*From the Clinical Research Laboratory  
Aided by grants from the Ophthalmological Foundation Inc.  
Received for publication July 21 1938

writers on the subject, although many of them, like Moore,<sup>5</sup> admitted the fallacy of the tests but concluded that they are "a very important factor as an aid to diagnosis of allergic diseases."

Much of the published data is difficult to interpret because of errors in bacteriologic diagnosis or the use of indefinite bacteriologic names. For example, one writer stated that 105 of his 133 strains of hemolytic streptococci were isolated from the gastrointestinal tract. It is more likely that many of these cultures were hemolytic enterococci, which are mostly nonpathogenic and possess properties distinct from other hemolytic streptococci. Other workers refer to any intestinal streptococcus as *Streptococcus fecalis* or to small, hemolytic surface colonies as hemolytic streptococci. Many of the latter may be alpha hemolytic or alpha prime streptococci, or may not even be streptococci.

Many of the published reports concerned work which was inadequately controlled. One writer reported using an average of only 3.6 tests per patient and 2.9 tests per control case.

Finally, the difference between the proportion of positive reactions in patients and in the controls does not show a marked contrast. In comparing patients with irritable colon with normal individuals, Mateer and Baltz<sup>6</sup> obtained diameters of 4.4 and 3.0 cm., respectively, with *B. coli communis*; 4.5 and 2.7 cm. with *B. coli communior*; 2.7 and 1.0 cm. with nonhemolytic streptococci; and 2.4 and 1.0 cm. with *Staphylococcus aureus*. The tests with *B. coli communis* were positive in 95 and 65 per cent, with *B. coli communior* in 96 and 69 per cent, with nonhemolytic streptococci in 62 and 21 per cent, and with *Staphylococcus aureus* in 49 and 50 per cent of patients and controls, respectively. Mateer and co-workers<sup>8</sup> obtained similar results.

Short, Dienes, and Bauer<sup>7</sup> maintained that variations in the skin reactions may be explained by differing irritability of the patients' skins, natural toxicity of the bacterial species, or possibly by a sensitization to certain bacterial groups.

Mateer and Baltz<sup>6</sup> claimed that the reaction decreased after immunization with the specific vaccine. However, their report indicates that the average reactions to *B. coli* were reduced only from 4.7 to 3.1 cm. in 12 patients immunized with *B. coli*. Steinberg and Wiltse<sup>2</sup> found an average area of 9.7 by 6.5 cm. before, and 3.9 by 2.3 cm. after immunization with *B. coli*.

Since certain in vitro tests have been shown to give results parallel with certain pathogenic properties of the cultures,<sup>9-15</sup> it was thought that these in vitro tests might be useful in comparing the results of intradermal tests. Agglutination tests were used for comparison also. A series of 305 smooth cultures was tested.

For the intradermal tests the bacteria were suspended in 1.0 per cent phenol in normal saline to make concentrations of 1 billion per c.c., and 0.05 c.c. was injected intradermally on the forearm. The results were read the following day.

The technique of the agglutination tests is described elsewhere.<sup>16</sup>

Pigment, hemolysis, and coagulase tests<sup>9</sup> were used as in vitro tests of staphylococci; resistance to the bactericidal action of fresh, diluted, defibri-

nated guinea pig blood<sup>10 11 15</sup> was used for streptococci, the electrophoretic migration velocity<sup>12 13</sup> was used for the colon group, and the crystal violet agar reaction<sup>14</sup> was used for *M. catarrhalis*.

TABLE I

RELATION BETWEEN INTRADERMAL TESTS, AGGLUTINABILITY AND IN VITRO TESTS OF PROBABLE PATHOGENICITY OF CULTURES ISOLATED FROM PERSONS SUSPECTED OF HAVING CHRONIC INFECTION

ORGANISM	CULTURES GIVING POSITIVE INTRADERMAL TESTS			CULTURES GIVING NEGATIVE INTRADERMAL TESTS			PER CENT AGREEMENT BETWEEN SKIN TESTS AND IN VITRO TESTS	PER CENT AGREEMENT BETWEEN SKIN TESTS AND AGGLUTINABILITY
	NUM. BEP. TESTED	IN VITRO POSITIVE	AGGLUTINABLE	NUM. BEP. TESTED	IN VITRO NEGATIVE	IN AGGLUTINABLE		
<i>B. coli</i>	10	2	5	11	10	10	40	50
<i>A. aerogenes</i>	9	5	2	2	0	2	45	36
Paracoli	4	0	0	4	4	2	50	25
Enterococci	4	2	2	19	10	10	91	91
Strep., gamma	7	0	1	14	12	14	70	88
Strep., alpha	11	5	1	105	55	65	54	57
Strep., beta	4	1	0	3	0	2	57	28
<i>M. catarrhalis</i>	1	0	1	5	5	5	80	100
<i>Staph. albus</i>	7	4	1	57	50	47	64	69
<i>Staph. aureus</i>	8	8	5	15	11	11	87	70

The results of the comparative tests are listed in Table I. There was agreement between intradermal and in vitro tests in 83 per cent of *M. catarrhalis* cultures, 84 to 87 per cent of staphylococci, and in 70 per cent of gamma type streptococci. Other groups showed agreement in less than 57 per cent of the tests.

There was agreement between intradermal tests and agglutinability in 91 per cent of enterococci, 88 per cent of gamma type streptococci, 100 per cent of *Micrococcus catarrhalis*, and 69 to 70 per cent of staphylococci. Other groups showed agreement in less than 57 per cent of tests.

#### CONCLUSIONS

Intradermal tests of bacteria isolated from patients suspected of having chronic infection were compared with (1) agglutination reactions of the strains using the serum of the person from whom the cultures were obtained and (2) in vitro tests which had been shown previously to have been parallel with certain pathogenic properties of the cultures.

The intradermal tests showed agreement with either agglutinability or the in vitro tests in more than 70 per cent of *Micrococcus catarrhalis*, staphylococci, gamma type streptococci, and enterococci.

There was less than 57 per cent agreement with either test in alpha and beta type streptococci, *B. coli*, *Acrobacter aerogenes*, and paracoli.

#### REFERENCES

1. Feinberg, S. M. The Uses and Limitations of Skin Tests in Allergy, *J. A. M. A.* 95: 1665, 1930.
2. Steinberg, B., and Wiltse, C. O. Skin Reactions to the Colon Bacillus and Its Toxic Products, *J. Immunol.* 22: 100, 1932.
3. Gow, M. H., and Herman, N. B. Intracutaneous Tests in Normal Individuals, An Analysis of 150 Subjects, *J. Allergy* 7: 108, 1936.

4. Solis-Cohen, M.: Comparison of the Relative Values of Intracutaneous Skin Test and of Pathogen-Selective Culture in Selecting Bacteria for Vaccines From Mixed Infections, *Am. J. Clin. Path.* 3: 305, 1933.
5. Moore, M. W.: The Value of Skin Testing as an Aid in the Diagnosis of Allergic Diseases, *Northwest Med.* 32: 224, 1933.
6. Mateer, J. G., and Baltz, J. I.: An Evaluation of Stool Vaccines in Chronic Irritable Colon Therapy, *Ann. Int. Med.* 5: 982, 1932.
7. Short, C. L., Dienes, L., and Bauer, W.: Autogenous Vaccines in Rheumatoid Arthritis, A Clinical Study and Critique, *Am. J. M. Sc.* 187: 615, 1934.
8. Mateer, J. G., Baltz, J. I., Fitzgerald, J., and Woodburne, H. L.: Colon Bacillus Vaccine Therapy, as Related to Chronic Functional Diarrhoea, Chronic Headache, Chronic "Toxic Vertigo" and "Unstable" Colon (Non-Ulcerative Colitis), *Am. J. Digest. Dis. & Nutrition* 2: 621, 1935.
9. Chapman, G. H., Berens, C., Peters, A., and Curcio, L.: Coagulase and Hemolysin Tests as Measures of the Pathogenicity of Staphylococci, *J. Bact.* 28: 343, 1934.
10. Rawls, W. B., and Chapman, G. H.: Experimental Arthritis in Rabbits, Comparison of the Arthritis-Producing Ability of Inagglutinable Streptococci Which Resist the "Bactericidal" Action of Fresh, Diluted, Defibrinated Guinea Pig Blood and Those Which Are Agglutinable but Sensitive to the "Bactericidal" Agent, *J. LAB. & CLIN. MED.* 21: 49, 1935.
11. Chapman, G. H., and Rawls, W. B.: Studies of Streptococci. I. Quantitative Differences in Resistance to Various Agents, *J. Bact.* 32: 323, 1936.
12. Chapman, G. H.: Electrophoretic Potential as an Aid in Identifying Strains of the *B. coli* Group, *J. Bact.* 18: 339, 1929.
13. Chapman, G. H., and Lieb, C. W.: Certain Electrophoretic Relationships of Colon Bacilli, *J. Bact.* In Press.
14. Chapman, G. H.: The Violet Agar Reaction as a Differential Characteristic of the *Micrococcus catarrhalis* Group, *Stain Technol.* 11: 25, 1936.
15. Chapman, G. H., Stiles, M. H., and Berens, C.: The Isolation and in Vitro Testing of Pathogenic Types of Non-exotoxic Streptococci, *Am. J. Clin. Path.* In Press.
16. Stiles, M. H., and Chapman, G. H.: Relationship Between Agglutinability and Certain In Vitro Tests of Staphylococci, Streptococci, and Colon Bacilli Isolated From Persons Suspected of Having Chronic Infection, *J. LAB. & CLIN. MED.* 24: 620, 1939.

---

## PRELIMINARY REPORT ON THE USE OF SULFANILAMIDE\*

E. F. TRAUT, M.D., AND C. E. LOGAN, M.D., CHICAGO, ILL.

---

**A**FTER making rather tentative use of sulfanilamide in a few cases of acute infections, and after noting its often spectacular effect, we studied the drug more intensively, in order to determine, if possible, the mechanism by which it acts, and the extent of its clinical possibilities.

This paper concerns the way sulfanilamide produces its therapeutic effect and the results of its use in a variety of acute and chronic infections. A drug may assist a patient in his battle against invading bacteria: (1) by making the patient's tissues an unsuitable culture medium, bacteriostasis; (2) by killing the bacteria, a bactericidal or bacteriolytic effect; (3) by increasing the number of leucocytes, and hence presumably the number of phagocytes; (4) by increasing the effectiveness of the existing phagocytes, enabling them to engulf and inactivate or kill a larger number of bacteria by increasing the opsonins in the patient's serum; (5) by inactivating the toxin produced by the bacteria, an antitoxic effect; (6) by making the tissues of the patient less sensitive or less susceptible to injury by toxin; (7) by causing the organism to assume less virulent forms, dissociation.

---

\*From the John McCormick Institute for Infectious Diseases, Chicago.  
Received for publication, July 23, 1938.

In this paper we report observations bearing definitely on the first, third, fourth, fifth, and seventh possibilities. The studies were made simultaneously on each patient before, during, and after treatment with prontosil (p amino phenylsulfonamide) and with "Prontosil (disodium 4 sulfamidophenyl 2 azo 7 acetyl amino 1 hydroxynaphthylene 3, 6 disulfonate) 25 per cent solution." These substances were furnished by the Department of Medical Research of the Winthrop Chemical Co. In our work the clinical effect was checked as far as possible bacteriologically.

**Phagocytosis**—Sulfanilamide induced a high degree of phagocytosis in experimental hemolytic streptococci peritonitis in mice according to Long and Bliss<sup>1</sup>.

Tunnicliff's method<sup>2</sup> of measuring phagocytosis and titrating opsonins was used in most instances. Occasionally it was necessary to use the older method with the patient's serum and indifferent leucocytes. The opsonins for the patient's own bacteria were titrated whenever the invaders could be isolated. In each instance the opsonins for strains of green streptococci recently isolated from the blood of a patient with acute arthritis and for a Type I pneumococcus (American Type Culture) were also determined. The opsonic index was measured before, during, and after treatment with prontosil or prontosil. The most positive increase in opsonic titer, after the use of sulfanilamide was observed in pneumonia. This increase of opsonic ability was coincident with improvement of the patient. It may be only a natural accompaniment of convalescence rather than a drug effect. Otherwise there was no suggestion that the drug affected phagocytosis in patients.

**Leucocytosis**—Daily leucocyte counts on patients and on three normal individuals before, during, and after treatment with prontosil and prontosil showed no variation other than the usual daily fluctuation.

**Inactivation of Bacterial Toxin**—Evidence is lacking to show any antitoxic tendency on the part of sulfanilamide or its compounds.

**Inhibition of Bacterial Growth**—Prontosil in vitro in a dilution of 1:100,000 markedly inhibited the growth of streptococci. *B. coli* from a stock culture showed no growth at 1:200,000, but a recently isolated strain grew well even in an 8 per cent solution. We have been able to grow streptococci only in dilutions higher than 1:20,000 and even then the growth was very sparse. According to Marshall, Emerson, and Cutting concentrations between 1:5,000 and 1:10,000 can be maintained in the blood of those treated. From the marked effect upon bacterial growth encountered in our in vitro experiments this would seem to be one of its important modes of action in infection.

**Dissociation**—Alterations of bacteria, chemical, antigenic, cultural, and morphologic, may be anticipated from a drug that so radically lessens the growth of bacteria in vitro. These changes in the bacteria due to growth in an unfavorable environment come under the heading of dissociation. They may at least in part, explain the favorable effect of sulfanilamide in disease. Such dissociation is suggested by one case in which green streptococci were isolated from the blood of a patient. After two weeks of intensive treatment with prontosil and prontosil, the blood flora had changed to indifferent streptococci. After one more week of treatment, cultures of the blood yielded no growth.



Another instance of this was found in a pneumonia patient. A green streptococcus was isolated in the first culture. After three days of prontosil, an indifferent organism was obtained, simultaneously with clinical improvement.

This report is concerned chiefly with the effect of sulfanilamide in pneumonia and arthritis. Patients with undulant fever, endocarditis, pneumococcus meningitis, and chronic cholangitis are also reported.

#### CLINICAL COURSE OF TREATED CASES

*Undulant Fever.*—The first patient treated for undulant fever was a 31-year-old male who had handled raw beef and pork for several years. For three weeks before admission to Cook County Hospital he had had generalized aching, chills, and fever. These symptoms persisted in the hospital, his fever rising to 103° every afternoon. The only significant abnormality was an enlarged spleen. The leucocyte count varied between 7,000 and 8,000. The Widal test was negative, as were also the cultures of blood and urine.

At the time of admission, the serum was found to agglutinate both the porcine and bovine types of *B. melitensis* through a dilution of 1:320 on the slide and above 1:1280 in the test tube. Semi-weekly injections of both forms of *B. melitensis* had brought no improvement after three weeks. For three more weeks the patient had only supportive treatment. Then he was given sulfanilamide, 40 grains for one day, and 30 grains daily thereafter for one week. The temperature was normal in forty-eight hours and did not rise again during a month of convalescence. He has had no recurrence after one year.

The second patient was a 69-year-old white female. She had been ill for two months with intermittent attacks of fever, vague rheumatic pains, and marked loss of weight and strength. Her hemoglobin was 50 per cent, red blood cells 2,500,000, and leucocytes 3,000. All tests for malaria and intestinal parasites were negative. Blood culture, however, was positive for *B. melitensis*, and her serum agglutinated this organism in a dilution of 1:15,000. When she became comatose a few days later, she was given a blood transfusion and 30 grains daily of prontosil. After forty-eight hours, she had a normal temperature and made an uneventful convalescence. She has remained well in the nine-month interval.

Berger and Schmetz<sup>4</sup> have reported recovery from Malta fever following administration of prontosil.

*Pneumococcal Infections.*—Nine pneumonia patients were given sulfanilamide in the Cook County Hospital. The mortality there is high because the patients are usually aged, alcoholic, or poorly nourished. During this year the mortality in the Cook County Hospital was about 50 per cent. There was no choice of patients treated with sulfanilamide. The patients were given 45 grains of sulfanilamide orally every twenty-four hours, and 20 c.c. of "prontosil soluble" intramuscularly every twelve hours. Two patients died. One of these, moribund on admission, lived only thirty-six hours.

The other patient had a bacteremia. Pneumococci in the sputum could not be typed by any of the available sera. He was very much improved, only to die suddenly during his convalescence. He was a marked alcoholic. During his treatment the blood organisms changed from greenish cocci to indifferent bacilli, either under the influence of sulfanilamide or as a natural accompaniment of convalescence.

Of the 7 (70 per cent) who fully recovered, 2 had a bacteremia.

The opsonins as measured by Tunnicliff's method increased sharply during the administration of sulfanilamide, either the result of the drug or part of a natural and expected increase in antibodies during the progress of an infection.

No immediate effect was seen which could be ascribed to a definite, prompt result of the drug in pneumonia.

A robust male of 45 years was given 10 cc injections of protosil every eight hours for four injections, thirty six hours after the onset of pneumococcal meningitis. The meningeal inflammation followed an acute otitis. He was in deep delirium when first seen. His spinal fluid was turbid and contained cells and innumerable pneumococci. He died sixty hours after the onset of his symptoms, without showing any good or bad effect from sulfanilamide administration.

*Arthritis*—Of great interest were the results in arthritis. In this group we treated only patients whom we could bacteriologically control.

One patient, a woman, has rheumatoid arthritis. Her elbows, wrists, knees, and fingers have been swollen and tender intermittently in long periods for five years. The joint symptoms are worse with occasional bouts of chills and fever. Repeated examinations for malaria and undulant fever (including skin tests with Brucella) have always been negative. Green streptococci have been isolated from her blood three times. Cultures of her colon taken according to our technique have repeatedly yielded from 50 to 98 per cent green streptococci in spite of repeated implantations and feedings of acidophilus bacilli, and of rapidly growing colon bacilli ("Mutaflo" Nissl). Colonic lavage improved her for a long time. In a flare up with an average temperature of 100° F and swollen knees, administration of 10 grains of sulfanilamide four times a day removed all signs of joint trouble and restored the temperature to normal within thirty six hours. It also changed the colonic flora from almost a pure culture of green streptococci to apparently complete absence of streptococci with a homogeneous growth of colon bacilli. The sulfanilamide was continued in doses of 5 grains three times daily for two weeks. Following this her colon remained relatively free of streptococci for three weeks. One month after discontinuing the drug the streptococcus count had risen to 50 per cent, and one week later the patient returned with an exacerbation of her joint disease.

Another woman of 59 years had had rheumatoid arthritis for years. Like the previous patient, all the usual foci had been removed. Green streptococci made up 70 to 90 per cent of her colonic flora at all times. She had a tendency to loose stools and abdominal distress. With these bowel upsets her joint symptoms were aggravated. Seven days after a course of 40 grains daily of sulfanilamide the streptococci had been completely eliminated from the colonic wall. Her joints were somewhat less painful but the drug induced a diarrhea controlled only by stopping it.

A woman of 72 years had had rheumatoid arthritis for eleven years. It had progressed to the point of marked disability. From one of her swollen knees turbid fluid was aspirated containing pleomorphic greening streptococci in pure culture. The culture of the blood was sterile. She took 30 grains of sulfanilamide daily for one week, and then 15 grains daily. Sixteen days after starting the drug the culture of the knee was sterile although a smear of the synovial fluid showed degenerated, poorly staining streptococcus forms. The knee joint has remained sterile. The arthritis has been very much relieved and the patient's general health is much improved. The sedimentation reading, 45 per cent in one hour at the onset of treatment, fell within four weeks to 18 per cent. She gained weight and strength. No other treatment was employed during the period of sulfanilamide administration.

A woman of 34 years, with active tuberculosis of the lungs and cervix, complained of pain in the joints. Blood culture yielded indifferent streptococci. Following three weeks of sulfanilamide, the blood culture was negative and has remained so. Her arthralgia and tuberculosis were unaffected.

*Cholangitis*—A woman of 42 years had suffered a complete stenosis of her common bile duct during or following cholecystectomy. A choledochogastric fistula developed spontaneously. The whole upper abdomen became a mass of dense adhesions discouraging further surgery. The woman felt ill. She had enough hyperbilirubinemia, without visible jaundice to produce pruritus. She complained of constant pain in the upper right abdomen and in the back. This sense of pressure increased during the day to become severe each afternoon with a chill and fever of 102° F. The paroxysm would be followed by profuse sweating. After carefully excluding malaria (by searching for malaria and administering quinine up to tolerance), undulant fever, gross hepatic abscess and pyelocystitis, the attacks were explained as Chreoc fever due to obstruction of the biliary passages with probably low grade infection of the

biliary tract, type undetermined. Cholagogues and duodenal drainage (Meltzer-Lyon technique) were without effect. Competent surgeons twice found the upper abdomen a mass of dense adhesions and located a fistula between the hepatic ducts and the stomach (cholechocho-gastric). The common duct could not be identified. The abdomen was closed without attempting the contemplated plastic surgery on the biliary ducts. Fever and chills continued. Administration of sulfanilamide 30 grains daily for three days resulted in complete disappearance of all symptoms. Her condition remained good during the medication and for two weeks after discontinuance of the drug. Recurrences were promptly checked by resuming the sulfanilamide in full dosage and eliminated by continuing the drug in daily doses of 1 gm.

*Endocarditis.*—Four patients with endocarditis received sulfanilamide. The course of two of these patients was apparently unaffected by the sulfanilamide. They died of cerebral embolism. Fever was eliminated from a third patient who later died of hemorrhage from a benign gastric polyp.

The other patient was 43 years old. He had had fever for ten weeks. He was very anemic, had petechiae and an enlarged spleen. He also had an enlarged heart with a systolic murmur. Greening streptococci were isolated from the blood. He ran a septic course for four weeks, becoming weaker daily. He became and remained afebrile after 60 grains of sulfanilamide. The drug was continued for three weeks. His blood became sterile and has remained so. After several weeks of observation, he left the hospital. He has returned since and successfully passed through a pneumonia. He remains well.

*Unfavorable Effects.*—Cyanosis attributable to the use of sulfanilamide has been observed in one instance. In this patient cessation of the drug "prontylin" and its substitution by oral "neoprontosil" in the same dosage resulted in freedom from the cyanosis. Complete blood counts, repeated at intervals of one to three days during the period of heaviest drug administration and weekly thereafter, have shown no changes not explained by the presence of infection. Frequent repeated urinalysis has never shown albumin, red blood cells, or casts suggesting renal irritation, nor detectable urobilinogen indicating liver injury.

Gastrointestinal irritation as manifested by nausea, burning, or diarrhea, has been the most frequent and most troublesome adverse effect.

#### SUMMARY AND CONCLUSIONS

Appreciating that too few patients have been observed, it is suggested that sulfanilamide may cure some patients of undulant fever.

Some patients subacutely or chronically infected by *Streptococcus viridans*, with arthritis or endocarditis, have been helped by the drug. Most interesting has been its effect upon the streptococci on the colonic mucosa.

Untoward effects have been limited to irritation of the gastrointestinal tract.

Bacteriostasis or limitation of bacterial growth and resultant limitation of injurious bacterial products as toxins, seems to us to be one probable mechanism of benefit by sulfanilamide in disease.

#### REFERENCES

1. Long, P. H., and Bliss, E. A.: Para-Amino-Benzene-Sulfonamide and Its Derivatives, J. A. M. A. 108: 32, 1937.
2. Tunnick, R.: The Identification of the Streptococcus of Scarlet Fever, J. A. M. A. 87: 625, 1926.
3. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: Para-Aminobenzenesulfonamide, J. A. M. A. 108: 953, 1937.
4. Berger, W., and Schnetz, H.: Prontosil in Abortus Infections, Med. Klin. 33: 594, 1937.
5. Traut, E. F., and Herrold, R. B.: A Simple Method of Making Cultures of the Rectum, J. Infect. Dis. 45: 172, 1929.

## A STUDY OF CALCIUM AND PHOSPHORUS IN CEREBROSPINAL FLUID\*

D. J. CONN, PH.D., I. KAPLAN, PH.D. AND A. LEVINSON, M.D. CHICAGO, ILL.

THE literature of cerebrospinal fluid calcium and phosphorus has been reviewed by Levinson and Katzenellenbogen.<sup>1</sup> The latter concluded that although the calcium content of the cerebrospinal fluid has been reported to be elevated in meningitis and some other diseases the number of instances of increased calcium was too small to be of diagnostic significance. The most complete work on phosphorus has been that of Cohen<sup>2</sup> who found the phosphorus content to be definitely elevated in tuberculous and meningococcal meningitis but not in acute epidemic encephalitis. This led Cohen to consider that the cerebrospinal fluid phosphorus may be of diagnostic importance in diseases of the nervous system. On the whole, the relationships between the calcium and phosphorus contents of the cerebrospinal fluid and the condition of the central nervous system are far from clearly defined. We found very few references to calcium and phosphorus in hydrocephalus and brain tumor. Our results may be of interest, therefore, since they indicate increased calcium and phosphorus values in tuberculous and purulent meningitis, hydrocephalus, and brain tumor.

Calcium determinations were made on 92 specimens of the cerebrospinal fluid, phosphorus determinations were made on 76 specimens. The specimens of cerebrospinal fluid were obtained from hospital patients, most of whom were children, at times when spinal punctures were necessary for diagnostic or therapeutic purposes. Lumbar punctures were made except where otherwise indicated. Calcium was determined by the Clark and Collip modification of the Kramer-Tisdall method.<sup>3</sup> Phosphorus was determined by the method of Bodansky,<sup>4</sup> except that in some cases 2 cc. of cerebrospinal fluid were used instead of 1 cc.

### RESULTS

*Group I Normal*—Group 1 contains fluids whose cellular and other chemical contents were normal. The calcium values of 20 fluids of this group were found to vary from 4.1 to 5.9 mg. per cent, with an average value of 5.0 mg. per cent. Only 4 fluids had values greater than 5.5 mg. per cent, and 3 had values less than 4.5 mg. per cent. These values are in close agreement with the values for normal fluids given by Katzenellenbogen<sup>1</sup> and Merritt and Fremont Smith,<sup>5</sup> and also for the values in epilepsy, idiocy, and cerebral birth trauma.

\*From the Department of Biochemistry, Nelson Morris Institute for Medical Research, the Children's Hospital of the Cook County Hospital, and the Sarah Morris Hospital for Children of the Michael Reese Hospital.

Alled by a grant from the Nelson Morris Fund.

Received for publication August 6, 1938.

TABLE I  
NORMAL CEREBROSPINAL FLUIDS

CASE	DIAGNOSIS	CELL COUNT	CALCIUM MG. PER CENT	PHOSPHORUS MG. PER CENT
B. B.	Epilepsy	1	5.0	1.3
R. P.	Epilepsy	3	4.5	1.4
J. B.	Epilepsy	4	4.7	
W. S.	Epilepsy	3	5.3	1.3
L. N.	Epilepsy	6	5.1	1.0
M. M.	Cerebral birth trauma	8	5.5	1.0
D. M.	Cerebral birth trauma	12	5.0	1.3
J. S.	Cerebral birth trauma	7	4.8	1.3
C. H.	Cerebral birth trauma	3	4.8	
A. F. (1)	Cerebral birth trauma	4	4.8	
A. F. (2)	Cerebral birth trauma		4.8	
N. J.	Cerebral agenesis	4	5.1	2.2
M. K. (1)	Cerebral agenesis	7	4.7	
M. K. (2)	Cerebral agenesis	0	5.7	1.5
S. S.	Idiocy	4	4.3	
D. R.	Idiocy	6	4.1	1.5
M. F.	Idiocy		4.4	1.2
B. A. F.	Idiocy		5.6	
B. B.	Idiocy	3	5.6	1.5
D. D.	Idiocy		5.9	1.1

TABLE II  
TUBERCULOUS MENINGITIS

CASE	APPEARANCE OF FLUID	CELL COUNT	CALCIUM MG. PER CENT	PHOSPHORUS MG. PER CENT
C. W.	Clear	150	7.2	
L. W. (1)	Clear	220	5.5	
L. W. (2)	Pink (Prontosil)	160	6.7	1.8
L. S. (1)	Clear	450	6.0	2.0
L. S. (2)	Clear	450	6.7	2.0
L. S. (3)	Clear	590	7.0	3.4
R. C.	Opalescent	350	6.9	1.4
E. T.	Clear		6.7	1.8
G. R. (1)	Clear	175	5.9	1.8
G. R. (2)	Opalescent	180	6.5	2.1
G. R. (3)	Opalescent	850		2.1
G. R. (4)	Opalescent	700	6.4	2.2
C. F.	Clear	98	6.2	3.0
L. C.	Clear	190	5.2	
M. W.	Opalescent		5.8	
I. W. (1)	Clear	126	5.3	1.6
I. W. (2)	Clear		5.6	1.8
*R. M. P.	Clear	160	5.4	1.4
F. C.	Opalescent	293	6.7	2.2
B. D.	Clear	200	6.9	1.8

\*Diagnosis not confirmed.

Thirteen fluids of this group were examined for their phosphorus content. In 12 fluids the phosphorus content lay between 1.0 and 1.5 mg. per cent; in one fluid a relatively high value of 2.2 mg. per cent was found. The average for the group was 1.4 mg. per cent. These values are also in close agreement with those given for normal fluids.<sup>1, 5</sup>

*Group II: Tuberculous Meningitis.*—The fluids from patients with tuberculous meningitis were generally found to have elevated calcium content. Nineteen fluids of this group were found to have calcium contents ranging from 5.2 to 7.2 mg. per cent, with an average value of 6.2 mg. per cent. Of these 19

fluids, 13 had values greater than 55 mg per cent, only 3 had values less than 55 mg per cent. It is of interest to note that when it was possible to obtain specimens of cerebrospinal fluid from a patient at different stages of the disease, the calcium values rose as the disease progressed. Thus, the calcium content of the fluid of patient L W rose from 55 to 67 mg per cent, that of L S, from 60 to 70, that of G R, from 59 to 65, and that of I W, from 53 to 56

TABLE III  
PURULENT MENINGITIS AND PYOCEPHALUS

CASE	DIAGNOSIS	APPEARANCE OF FLUID	CELL COUNT	CALCIUM MG PER CENT	PHOS- PHORUS MG PER CENT
R W	Influenzal	Very turbid	5000 poly	59	
R H	Influenzal	Very turbid		49	
W G (1)	Streptococcic (anhemolytic)	Very turbid	600 poly	65	
W G (2)	Streptococcic (anhemolytic)	Very turbid, ventricular	4600 poly		15
W G (3)	Streptococcic (anhemolytic)	Very turbid	5000 poly		30
G O (1)	Streptococcic (hemolytic)	Very turbid	8500 poly	63	
G O (2)	Streptococcic and viridans	Very turbid	720 poly	64	
J D (1)	Streptococcic (hemolytic)	Very turbid	2200 poly	56	
J D (2)	Streptococcic (hemolytic)	Opalescent	440 poly	45	
J B	Staphylococcic	Turbid	6000 poly		12
J J	Pneumococcic	Turbid	5000 poly		12
D R (1)	Meningococcic	Turbid	3000 poly	52	14
D R (2)	Meningococcic	Turbid	1200 poly	49	14
D R (3)	Meningococcic	Opalescent	45 lymph	40	21
K S (1)	Influenzal	Very turbid	2800 poly		19
K S (2)	Influenzal	Opalescent	180 poly	60	18
K S (3)	Influenzal	Opalescent	1650 poly	60	
*R K (1)	B pyocyaneus	Very turbid, ventricular, frank pus		66	70
R K (2)		Very turbid, ventricular, frank pus		87	
†R K (3)	B pyocyaneus	Very turbid, ventricular, frank pus		103	74
R K (4)	B pyocyaneus	Very turbid, ventricular, frank pus		123	92

\*The cerebrospinal fluid protein values were very high in case (R K) being 2500-2930 and 3810 mg per cent in (1), (3) (4), respectively.

†The values of the serum calcium and phosphorus were normal being 10.3 and 5.0 mg per cent respectively.

The phosphorus content of the fluids of this group, like that of the calcium was generally elevated. The values found for 16 fluids ranged from 1.4 to 3.0 mg per cent, with an average of 2.0 mg per cent. In only 2 fluids was the phosphorus content less than 1.5 mg per cent, in 7 it was 2.0 mg per cent or higher. The phosphorus content of the cerebrospinal fluid also seemed to rise as the disease progressed. In one case, L S, it rose from 2.0 to 3.4 mg per cent, in the case of G R, from 1.8 to 2.2 mg per cent, and in the case I W, from 1.6 to 1.8 mg per cent.

*Group III Purulent Meningitis and Pyocephalus*—The fluids of this group were also found to be higher in calcium and phosphorus than the normal fluids. Of 16 fluids studied, 12 from patients with meningitis were found to range in

TABLE IV  
HYDROCEPHALUS

CASE	APPEARANCE OF FLUID	CELL COUNT	CALCIUM MG. PER CENT	PHOSPHORUS MG. PER CENT
W. H. (1)	Opalescent, ventricular	310 Poly.	7.6	5.2
W. H. (2)	Opalescent, ventricular	350 Poly.	7.5	6.0
G. C.	Xanthochromic, ventricular		6.1	
W. W.	Xanthochromic, lumbar	40 Lymph.	6.9	2.6
B. S. (1)	Clear, ventricular		7.5	1.8
B. S. (2a)	Clear, lumbar	30 Lymph.	6.2	1.7
B. S. (2b)	Clear, ventricular	10 Lymph.	6.1	2.0
M. R. (1)	Clear, lumbar	20 Lymph.	5.2	2.1
M. R. (2)	Clear, lumbar	1 Lymph.	5.4	2.1
J. M.	Clear, ventricular	3 Lymph.	6.5	2.9
R. G. (1)	Clear, lumbar	2 Lymph.	4.9	
R. G. (2)	Clear, lumbar	2 Lymph.	4.4	1.2
N. L. W.	Clear, ventricular		6.3	1.2
B. G. B. (1)	Clear, ventricular		7.4	
B. G. B. (2)	Clear, ventricular		7.0	
B. B. L.	Xanthochromic, ventricular		7.6	

TABLE V  
BRAIN TUMOR, BRAIN ABSCESS, BRAIN CYST

CASE	DIAGNOSIS	APPEARANCE OF FLUID	CELL COUNT	CALCIUM MG. PER CENT	PHOS- PHORUS MG PER CENT
J. M.	Cerebellar Ghoma	Clear, ventricular	3 Lymph.	6.5	2.9
*E. P. (1)	Glioblastoma of left temporal lobe	Clear, lumbar	120 Lymph.	6.9	2.7
E. P. (2)	Glioblastoma of left temporal lobe	Clear, lumbar	150 Lymph.	6.7	2.2
E. P. (3)	Possible subarachnoid block	Yellow, lumbar, viscid	450 Red blood cells		1.7
E. P. (4)	Possible subarachnoid block	Yellow, lumbar, viscid		8.3	3.9
E. P. (5a)	Possible subarachnoid block	Yellow, lumbar, viscid	7 Lymph. Some red blood cells		4.5
†E. P. (5b)	Possible subarachnoid block	Clear, subarachnoid ?	7 Lymph.	5.6	1.6
†E. P. (6a)	Possible subarachnoid block	Yellow, lumbar, viscid		8.3	4.0
†E. P. (6b)	Possible subarachnoid block	Subarachnoid ?	Some red blood cells	4.6	2.0
W. W.	Cerebellar medulloblas- toma	Xanthochromic	40 Lymph.	6.9	2.6
C. K.	Cerebral abscess	Turbid	3000 Poly.		1.2
L. C. (1)	Cerebral abscess	Clear	170 Lymph.	5.6	1.1
L. C. (2)	Cerebral abscess	Clear	22 Lymph.		1.5
R. M. (1)	Cerebral subarachnoid cyst	Xanthochromic	0 Lymph.	5.0	
R. M. (2)	Cerebral subarachnoid cyst	Xanthochromic	110 Lymph.	6.6	
R. M. (3)	Cerebral subarachnoid cyst	Xanthochromic			1.9
R. M. (4)	Cerebral subarachnoid cyst	Xanthochromic		6.4	2.1

\*The cerebrospinal fluid protein values for case (E. P.) were 207, 154, 3600, 2760, 3570, 25, 3930, and 27 for fluids 1, 2, 3, 4, 5a, 5b, 6a and 6b, respectively.

†From decompressed area above tumor.

‡The values of the blood serum calcium and phosphorus were 9.6 and 6.1 mg per cent respectively, i.e., normal.

value from 4.0 to 6.5 mg per cent, with an average of 5.5 mg per cent. In two instances in which patients recovered, J. D. and D. R., the calcium values decreased from 5.6 to 4.5 mg per cent, and from 5.2 to 4.0 mg per cent, respectively.

Phosphorus, in 9 fluids from patients with meningitis, ranged in value from 1.2 to 3.0 mg per cent, with an average value of 1.7 mg per cent.

Four fluids were obtained from patient R. I. with *B. pyocyaneus* pyocephalus. The calcium values found were 6.6, 8.7, 10.2, and 12.3 mg per cent, increasing as the disease progressed. Phosphorus, determined in 3 fluids, had values of 7.0, 7.4, and 9.2 mg per cent. Although on autopsy no meningeal exudate was found, pathologically the fluids closely resembled those of the purulent meningitides. For this reason they are included in group III.

**Group IV. Hydrocephalus.**—The calcium content of fluids from patients with hydrocephalus was found to show marked elevations. Sixteen fluids from 10 patients had values from 4.4 to 7.6 mg per cent, with an average of 6.4 mg per cent. Only 4 fluids from 2 patients with mild hydrocephalus fell within the range of normal values.

Phosphorus was determined in 11 fluids from 7 patients. Values ranged from 1.2 to 6.0 mg per cent, with only two values within the normal range. The average value for the group was 2.6 mg per cent.

**Group V. Brain Tumor, Brain Abscess, Brain Cyst.**—Most of the fluids in this group, like those of group IV, had elevated calcium and phosphorus contents. Twelve fluids were examined for calcium content and were found to have values ranging from 5.0 to 8.3 mg per cent, with an average of 6.4 mg per cent. In only two instances were the values less than 5.5 mg per cent. A specimen obtained at a later date from patient R. M. showed an increase from 5.0 to 6.6 mg per cent.

Phosphorus was determined in 15 fluids of this group, the values ranged from 1.1 to 4.5 mg per cent, with an average of 2.4 mg per cent. Eleven fluids of the 14 had values greater than 1.5 mg per cent.

TABLE VI  
MISCELLANEOUS

CASE	DIAGNOSIS	CELL COUNT	CALCIUM MG PER CENT	PHOSPHORUS MG PER CENT
I. W.	Cerebral arteriosclerosis	4	5.2	1.4
II. K.	Hemihypertrophy of the body	"	4.0	0.9
II. W.	Chorea	4	4.9	1.5
A. M. B.	Chorea	6	4.6	1.4
L. M.	Postencephalitis syndrome	12	5.8	1.2
T. W.	Postdiphtheritic rabies	8	4.8	
F. I.	Acute benign lymphocytic meningitis	290		1.2
L. T.	Preparalytic poliomyelitis	120		1.1
D. M.	Poliocencephalitis ?	9	5.1	1.6
K. B.	"	170	5.4	
I. W.	"		4.6	1.8
A. W.	"	200	5.1	0.8
C. T.	"	87		1.2
C. M.	Postmenstrual encephalitis	1		1.0
B. S.	Postmenstrual encephalitis	1	6.0	1.4



*Group VI: Miscellaneous.*—On the whole, the calcium and the phosphorus contents of the fluids of this group resembled those of normal fluids. The calcium values of 11 fluids ranged from 4.0 to 6.0 mg. per cent, with an average of 5.0 mg. per cent. In only two instances was the value greater than 5.5 mg. per cent. Phosphorus values, determined from 13 fluids, ranged from 0.8 to 1.8 mg. per cent, with an average of 1.3 mg. per cent.

TABLE VII

	CALCIUM MILLIGRAMS PER CENT			PHOSPHORUS MILLIGRAMS PER CENT		
	<i>Minimum</i>	<i>Maximum</i>	<i>Average</i>	<i>Minimum</i>	<i>Maximum</i>	<i>Average</i>
Group I						
Normal	4.1	5.9	5.0	1.0	2.2	1.4
Group II						
Tuberculous meningitis	5.2	7.2	6.2	1.4	3.0	2.0
Group III						
Purulent meningitis	4.0	6.5*	5.5*	1.2	3.0*	1.7*
		12.3†	7.1†		9.2†	3.3†
Group IV						
Hydrocephalus	4.4	7.6	6.4	1.2	6.0	2.6
Group V						
Brain tumor	5.0	8.3	6.4	1.1	4.5	2.4
Brain abscess						
Brain cyst						
Group VI						
Miscellaneous	4.0	6.0	5.0	0.8	1.8	1.3

\*Excluding the values for fluids from patient (R.K.).

†Including the values for fluids from patient (R.K.).

## DISCUSSION

The results of our work indicate that the cerebrospinal fluid calcium and phosphorus are generally elevated in tuberculous and purulent meningitis, hydrocephalus, brain tumor, and brain cyst. Some of the possible causes of these increases will be discussed briefly.

Probably the chief cause of increased cerebrospinal fluid calcium and phosphorus in meningitis is the increased meningeal permeability caused by the infection. It is possible to understand on this basis the increase in the calcium and phosphorus values with the progress of the disease. Thus an increase in the length of time, during which the meningeal permeability has been elevated, tends to increase the amounts of calcium and phosphorus reaching the subarachnoid space from the blood. The opposite may be the case if the progress of the disease is arrested and the patient recovers. However, it is difficult to understand why, in some cases of purulent meningitis (Table III, R. H., J. D., D. R.), the calcium values are not elevated, even though on recovery (J. D., D. R.) the values did decrease. Similarly, the phosphorus values were sometimes normal in severe cases of purulent meningitis. Also it is interesting to note that the calcium and phosphorus values were more consistently high in tuberculous meningitis than in purulent meningitis. This may be due to the fact that the disease ran a longer course in the case of tuberculous meningitis than in purulent meningitis. It is possible that other factors besides increased meningeal permeability are involved. It has been reported<sup>6,7</sup> that in cases of tuberculous meningitis, there is often an extension of the inflammatory process into the cortex, resulting in foci of encephalitis. Whether the resulting brain

tissue destruction may be related to the consistently elevated calcium and phosphorus values of tuberculous meningitis is as yet an open question.

The fluids from patients with hydrocephalus and brain tumor fell into two groups, those which were xanthochromic and had high protein content, and those which were clear and relatively low in protein content. The fluids of the former group all had high calcium content, some of the fluids of the latter group were also high in calcium content, others were normal. Xanthochromic fluids were obtained from patients who were found on autopsy to have had lesions which invaded the meninges or ventricles. Walter<sup>8</sup> has reported that in such cases there is increased meningeal permeability. In the fluids of this type, the high calcium and phosphorus content may also be due in part to brain tissue destruction.

The mechanism by which the cerebrospinal fluid calcium and phosphorus may be increased during the course of brain tissue destruction is not clear. Recent work<sup>9</sup> indicates that the calcium content of fresh normal brain tissue is about 4 mg per 100 gm. The complete degeneration of 100 gm of brain tissue could have no significant effect on the calcium content of the cerebrospinal fluid, if those figures are correct. It follows that the brain tissue cannot be the source of the increase in cerebrospinal fluid calcium. It is known, however, that degeneration of tissue is often followed by calcification. Wells<sup>10</sup> stated it as a rule that, except for the metastatic calcification of Vinchow, degeneration always precedes pathologic calcification. The calcium salts involved come from the blood vessels of the degenerating tissue. One example of this type of pathologic calcification is that found in ganglion cells of the brain which have become degenerated or necrotic. Further, Gevelin and Penfield<sup>11</sup> have reported the occurrence of a group of cases of familial cerebral calcification with epilepsy. They considered that the pathologic process underlying the group of cases was a slowly progressive closure of the end arteries in the cerebrum which produced an area of local necrosis which then become calcified. Whether calcification actually does occur in an area of degeneration or not, it is possible that there is an accumulation of calcium salts in that area. Some of those salts may be carried to the subarachnoid space via the perivascular spaces. In cases where there is no evidence of increased meningeal permeability, the increased calcium content of the cerebrospinal fluid may be an index of calcium salt accumulation in an area of brain tissue degeneration. This hypothesis is in agreement with the fact that in cases of severe hydrocephalus with much cortical destruction high calcium values were found, in cases where encephalography showed relatively slight degeneration, calcium values were normal.

The phosphorus content of fresh normal white matter is, according to Alexander and Myerson,<sup>9</sup> about 530 mg per 100 gm, that of gray matter is about 180 mg per 100 gm. Since cerebral pressure atrophy involves chiefly the white matter,<sup>12</sup> the destruction of large amounts of brain tissue could contribute significantly to the phosphorus content of the cerebrospinal fluid. It is probable that some phosphorus accompanies the calcium which comes from the blood vessels. There are, therefore, two possible sources of phosphorus, so that together with increased calcium values, increased phosphorus content of the cerebrospinal fluid may be an index of brain tissue destruction.

The clinical usefulness of our results cannot be definitely evaluated as yet. In tuberculous meningitis the elevation of calcium and phosphorus, together with other chemical findings, may be of diagnostic help. In purulent meningitis the calcium and phosphorus values are not sufficiently consistent to be of much aid clinically. In hydrocephalus and brain tumor high calcium and phosphorus values may indicate brain tissue destruction and may be of prognostic value in those conditions.

#### CONCLUSIONS

1. The calcium content of normal cerebrospinal fluid was found to vary from 4.5 to 5.5 mg. per cent, with an average of 5.0 mg. per cent. The phosphorus content varies from 1.0 to 1.5 mg. per cent.

2. Calcium and phosphorus were found to be consistently elevated in tuberculous meningitis, the values increasing with the progress of the disease.

3. Calcium and phosphorus values were occasionally elevated in purulent meningitis, but not so consistently as in tuberculous meningitis.

4. Calcium and phosphorus were generally elevated in hydrocephalus and in brain lesions.

5. The causes of elevated calcium and phosphorus values are probably increased meningeal permeability and brain tissue destruction. The latter may be of particular interest in hydrocephalus and brain tumor.

6. The elevation of cerebrospinal fluid calcium and phosphorus may be of corroborative value in the diagnosis of tuberculous meningitis and may yield some information about the destruction of brain tissue in hydrocephalus and brain tumor.

#### REFERENCES

1. Levinson, A.: *The Cerebrospinal Fluid in Health and in Disease*, St. Louis, 1929, C. V. Mosby Co.
- Katzenellenbogen, S.: *The Cerebrospinal Fluid and Its Relation to the Blood*, Baltimore, 1935, The Johns Hopkins Press.
2. Cohen, H.: The Inorganic Phosphorus Content of the Cerebrospinal Fluid, *Quart. J. Med.* 17: 289, 1924.
3. Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium With a Suggested Modification, *J. Biol. Chem.* 63: 461, 1925.
4. Bodansky, A.: Phosphatase Studies. I. Determination of Inorganic Phosphate. Beer's Law and Interfering Substances in the Kuttner-Lichtenstein Method, *J. Biol. Chem.* 99: 197, 1932-1933.
5. Merritt, H. H., and Fremont-Smith, F.: *The Cerebrospinal Fluid*, Philadelphia, 1937, W. B. Saunders Co.
6. Beres, D., and Meltzer, T.: Tuberculous Meningitis and Its Relation to Tuberculous Foci in the Brain, *Am. J. Path.* 14: 59, 1938.
7. Levinson, A.: Meningitis Masked by Encephalitis Symptoms, *Arch. Pediat.* 49: 6, 1932.
8. Walter, F. K.: *Die Blut-Liquor Schranke*, Leipzig, 1929, Georg Thieme.
9. Alexander, L., and Myerson, A.: Minerals in Normal and Pathologic Brain Tissue, Studied by Microincineration and Spectroscopy, *Arch. Neurol. & Psychiat.* 39: 131, 1938.
10. Wells, H. G.: *Chemical Pathology*, ed. 5, Philadelphia, 1925, W. B. Saunders Co.
11. Geyelin, H. R., and Penfield, W.: Familial Calcifying Cerebral Degeneration, *Arch. Neurol. & Psychiat.* 21: 1020, 1929.
12. Penfield, W., and Elvidge, A. R.: Hydrocephalus and the Atrophy of Cerebral Compression. *Cytology and Cellular Pathology of the Nervous System*, Vol. III, New York, 1932, Paul B. Hoeber.

## A PRELIMINARY REPORT OF THE BLOOD PICTURE IN BRUCELLOSIS\*

MARTIN MINGIE, B.S., and I. FOREST HENDERSON, PH.D.  
EAST LANSING, MICH.

THERE are scattered reports in the literature to the effect that the blood picture in undulant fever is characterized by a leucopenia with a relative lymphocytosis,<sup>1, 2</sup> a slight to moderate secondary anemia, and a color index of less than one.<sup>1</sup> There has been no extensive study of the blood picture in many cases of undulant fever from which one of the species of *Brucella* was isolated.

An opportunity presented itself this year to study the blood picture on a number of acute undulant fever cases from which *Brucella melitensis* was recovered. The patients were located on the island of Malta. All patients were showing symptoms of the disease at the time of examination. The blood examinations were made from two to four weeks after the onset of the disease.

The data from 10 cases in Table I are representative of the findings from 32 cases studied.

**Size of Red Blood Cells**—We have been unable to find any references pertaining to the size of the red blood cells in undulant fever. A study of their size in these cases revealed that there is a marked variation in the blood of undulant fever patients from the normal. In 35 per cent of the patients studied it was found that an increased number of the cells were smaller than normal and in 19 per cent there was a tendency of the cells to be larger than normal. The average red blood cell measurement formula was of a 39.37:24 ratio instead of the 33.34:33 ratio<sup>3</sup> which we are using as the normal distribution. The red blood cells of some patients varied in size from 3 to 10 microns, although the normal variation is from 6 to 10 microns. This variation in size is probably effected by an accompanying splenic and liver pathology.

**White Blood Cells**—The white blood cell count on the average is lower than normal with a relative and absolute monocytosis.<sup>3</sup> Qualitatively we find an increase of the nonfilamented neutrophils. For several hours after injections of brucellin (the curative agent) there is an elevation of the leucocyte count due to the increase of the nonfilamented and filamented neutrophils. The blood picture does not return to normal until recovery has taken place.

One interesting point that has not been noted before is that many of the mature small lymphocytes are much larger than normal which is similar to the infectious mononucleosis type of lymphocyte. These have been termed by Calder<sup>4</sup> "pathologic lymphocytes," and others have named them "large mature lymphocytes." These cells have all the appearance of small lymphocytes, the ratio of the nucleus to cytoplasm being similar to that of the normal small lymphocyte. They are about 12 to 14 microns in diameter. The normal small

\*Journal article No. 333 ns. From the Michigan Agricultural Experiment Station. This study was aided by a grant from the Horace H. and Mary A. Richman Fund.

Received for publication August 8, 1938.

TABLE I

CASE NO.	W. R. C.	DIFFERENTIAL COUNT PER CENT						LVD*	BGN*	RED BLOOD CELL MEASUREMENT		
		EOSINO- PHILES	BASO- PHILES	NONTUA- MENTED NEUTRO- PHILES	PLA- MENTED NEUTRO- PHILES	LARGE LYMPHO- CYTES	SMALL LYMPHO- CYTES	MONO- CYTES		7.5 $\mu$ <	7.5 $\mu$	7.5 $\mu$ >
3 (2)	5,000	1	2	12	16	49	10	10	44	21	38	41
6 (1)	4,636	0	0	21	10	19	1	17	34	31	34	15
7 (1)	5,200	0	0	29	6	47	7	11	24	45	55	19
9 (2)	4,610	0	0	27	12	49	6	6	34	38	43	22
12 (1)	5,025	0	0	30	27	31	4	8	24	75	26	5
13 (1)	4,100	0	0	30	23	36	4	7	34	27	32	21
15 (1)	2,800	0	0	26	23	38	6	6	44	31	40	26
17 (1)	3,300	0	0	31	20	40	2	7	34	31	39	27
17 (2)	3,500	0	0	15	30	40	4	11	24	33	31	33
18 (2)	4,900	0	0	31	21	36	3	9	14	32	33	35
Average	3,950	0.1	0.2	25.2	18.8	41.4	5.0	9.2		39.0	37.0	24.0
Normal	8,000	1	0.5	6	60	8	20	4.5	0	33	31	33

\*LVD = Liver damage cell.

BGN = Basophilic granulation of the neutrophils.

lymphocytes usually measure from 8 to 10 microns. As many as 30 to 80 per cent of the lymphocytes may appear as this type of large cell in about 40 per cent of the brucellosis patients.

Sabin<sup>5</sup> by supravital staining has found, in cases of brucellosis, an increase in the type of monocyte which is similar morphologically to the type of monocytes associated with various forms of hepatic involvement.<sup>6</sup> These appear to be similar to the atypical monocytes found in catarrhal jaundice.<sup>7</sup> In disease associated with liver pathology, Isaacs<sup>7</sup> has described "a cell averaging 15 by 13 microns, with an oval nucleus, rather dense chromatin (lymphoid in character), foamy blue staining cytoplasm (monocytoid), but with absence of the minute, red staining granules of the monocyte. There is no perinuclear clear zone, as in the lymphocyte. Occasional inclusion granules are found in the cytoplasm. The margin is wavy." The "liver damage cell" of Isaacs was present consistently in all of the cases of brucellosis examined.

Another interesting point that has been noted in these Maltese patients is that in *Brucella melitensis* infection there is a marked basophilia of the granules of the neutrophils. This phenomenon may be associated with the temperature elevation that the patients experienced. We believe that this is characteristic of the *Brucella melitensis* infection, since we have not encountered basophilia of the granules in *Brucella suis* and *Brucella abortus* infections. These granules are similar in size to the *Brucella* bacteria and stain similarly. It is suggested that a control plain smear always be made without the bacteria when a phagocytosis test is performed. A toluidine blue staining technique has been perfected by the junior author for the staining of phagocytosis smears from *Brucella melitensis* infected patients.

#### SUMMARY AND CONCLUSION

We have found in the study of *Brucella melitensis* infected individuals that the blood picture reveals a leucopenia with a relative lymphocytosis and monocytosis.

The red blood cells tend to be slightly smaller than normal, however, some patients gave evidence of macrocytosis.

The presence of "pathologic lymphocytes" in 40 per cent of the brucellosis cases is significant.

"Liver damage cells" were found consistently in these patients.

Finally the basophilia of the granules of the neutrophils seems to differentiate the *Brucella melitensis* infection from that of the *suis* and the *abortus* infection.

#### REFERENCES

- 1 Kriele and Carver. Disease of the Blood and Atlas of Hematology, Philadelphia, 1937, J. B. Lippincott Co., pages 74, 125.
- 2 Pepper and Farley. Practical Hematological Diagnosis. Philadelphia, 1934, W. B. Saunders Co., page 503.
- 3 Runsford, S. G. J. Roy. Nav. Med. Serv. 19, 1933.
- 4 Calder, Royall M. Personal communication.
- 5 Sabin, F. R. Studies of Living Human Blood Cells, Bull. Johns Hopkins Hosp. 34, 277, 1934.
- 6 Downey, Hal. Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., Vol. 1, pages 613-616.
- 7 Isaacs, Raphael. Personal communication.
- 8 Weiss, H. B., and Isaacs, R. Manual of Clinical and Laboratory Technique, ed. 5, Philadelphia, 1937, W. B. Saunders Co., page 54.

# RELATIONSHIP BETWEEN AGGLUTINABILITY AND CERTAIN IN VITRO TESTS OF STAPHYLOCOCCI, STREPTOCOCCI, AND COLON BACILLI ISOLATED FROM PERSONS SUSPECTED OF HAVING CHRONIC INFECTION\*

MERRITT H. STILES, M.D., PHILADELPHIA, PA., AND GEORGE H. CHAPMAN  
NEW YORK, N. Y.

**A**GGLOTINATION reactions have been used in attempts to appraise the significance of bacteria isolated from suspected foci of infection. In efforts to determine the reliability of these reactions it was reasoned that pathogenicity of the organisms should play a part in their relationship to the disease process. However, it would have been impractical to make animal inoculation tests on the twelve thousand cultures of staphylococci, streptococci, and colon bacilli used in these experiments. Therefore, the following tests were used because they had previously been shown to have given results parallel with certain pathogenic properties of the cultures. Pigment, hemolysis, and coagulase tests<sup>1</sup> were used for staphylococci; resistance to the bactericidal action of fresh, diluted, defibrinated guinea pig blood<sup>2-4</sup> was used for streptococci; and the electrophoretic migration velocity<sup>5, 6</sup> was used for the colon group. These will be referred to as in vitro tests.

For the agglutination reactions, pure cultures of streptococci were grown in brain heart infusion overnight, tested for purity, washed once with 1.0 per cent phenol in 0.85 per cent salt solution, and resuspended in it. The final concentrations were about 10 billions per c.c. Other bacteria were grown on solid media and suspended in the phenol saline without washing. Unless the suspensions were decanted before use, the control tubes often contained a deposit of bacteria and extraneous matter which was difficult to distinguish from specifically agglutinated particles. The methods described by Spicer<sup>7, 8</sup> and Mueller and Klise<sup>9</sup> were found useful for preparing suspensions of auto-agglutinative cultures.

A series of tubes containing 1.0 c.c. of progressive dilutions of the patient's serum, ranging from 1:40 to 1:5,120, together with a saline control, were placed in a rack. To each tube was added 1.0 c.c. of the bacterial suspension. After being shaken for two minutes, the tubes were placed in a water bath at 50° to 55° C. for one hour. The tubes were inspected periodically, and the results read whenever the control tube began to show excessive sedimentation. Otherwise, the rack was left on the laboratory table until the following morning, and the results read at that time.

\*From the Clinical Research Laboratory, New York, N. Y.  
Aided by grants from the Ophthalmological Foundation, Inc.  
Received for publication, July 21, 1938.

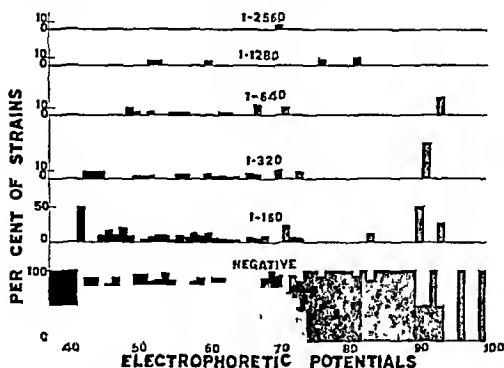


Fig 1—Comparison of electrophoretic migration velocity and agglutinability of 593 *B. coli-typhosa* cultures

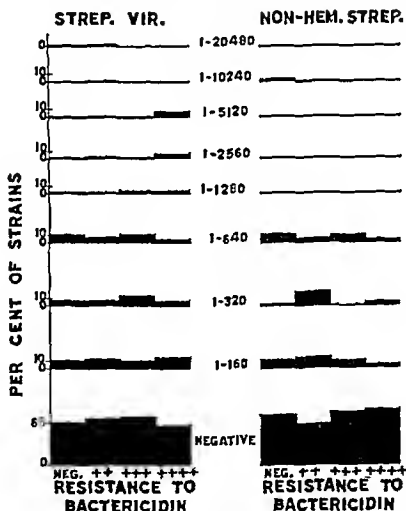


Fig 2—Comparison of resistance to the bactericidal action of fresh, diluted defibrinated guinea pig blood and agglutinability of 627 cultures of *Streptococcus viridans* and 107 cultures of gamma type streptococci

Some of the tests gave an increased titer when re-examined. This was found to have been caused by jarring the tubes when they were dropped back into the racks. In subsequent tests the tubes were lifted about 1 cm. and dropped back into the racks, and this was repeated a short while later. The results were read soon afterward. Because many of the sera agglutinated most of the organisms in dilutions up to 1:80, only reactions of 1:160 and over were considered positive.



Certain sera agglutinated a wide variety of bacteria, some of them in high dilution. Other sera gave only weak reactions. This suggested that agglutination was as much a function of the serum as of the bacteria.

The proportion of agglutinable cultures in different groups was as follows: *Staphylococcus aureus*, 47 per cent; hemolytic streptococci, 42 per cent; *Streptococcus viridans*, 36 per cent; *Staphylococcus albus*, 32 per cent; gamma type

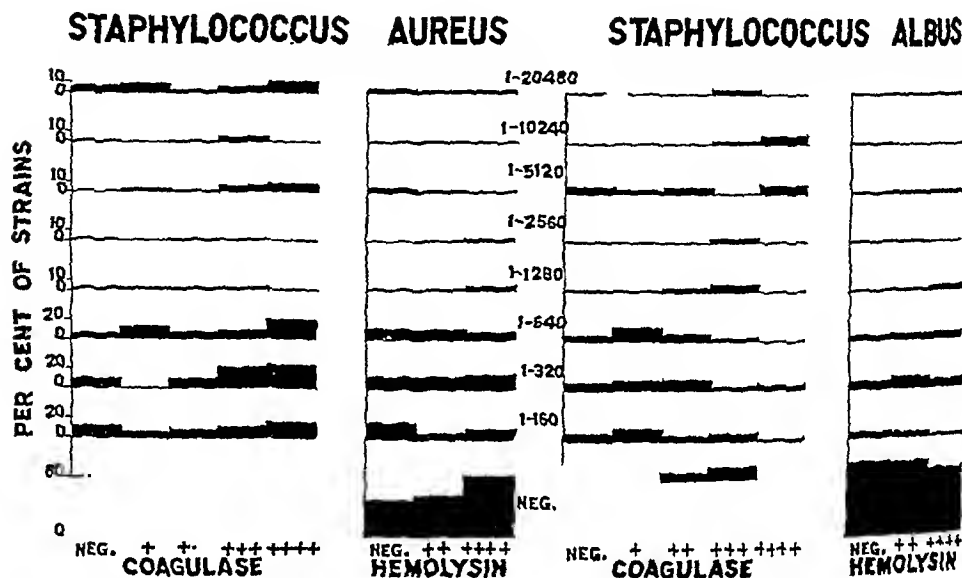


Fig. 3.—Comparison of hemolytic and coagulating properties with the agglutination titer of 368 cultures of *Staphylococcus aureus* and 885 cultures of *Staphylococcus albus*.

streptococci, 28 per cent; gamma type enterococci, 25 per cent; colon bacilli, 14 per cent; and alpha type enterococci, 10 per cent. In a series of 110 normal adults, Steinberg and Wiltzie<sup>10</sup> obtained with colon bacilli a titer of 1:100 in 3, 1:300 in 4, and 1:1,000 in 1 person.

TABLE I

RELATIONSHIP BETWEEN AGGLUTINABILITY AND IN VITRO TESTS OF BACTERIA ISOLATED FROM PERSONS SUSPECTED OF HAVING FOCAL INFECTION

TYPE OF ORGANISM	CULTURES EX-AMINED	IN VITRO POSITIVE		IN VITRO NEGATIVE		PER CENT AGREEMENT (COLUMNS 1 + 4)
		AGGL. + (1)	AGGL. 0 (2)	AGGL. + (3)	AGGL. 0 (4)	
<i>Staphylococcus albus</i>	2975	7.2	11.3	16.3	65.3	72.5
<i>Staphylococcus aureus</i>	1122	52.3	40.6	3.5	3.7	50.0
Hemolytic streptococci	309	26.4	32.3	21.3	19.7	46.1
Nonhemolytic streptococci	861	13.8	29.8	16.7	39.7	53.5
<i>Streptococcus viridans</i>	4980	19.5	39.9	18.2	22.8	42.3
Colon bacilli	2148	1.9	20.4	12.2	65.5	67.4

Reactions considered positive:

*Staphylococci*—any strain which coagulated human and rabbit plasma.

*Streptococci*—cultures giving +++ and +++ resistance to fresh, diluted defibrinated guinea pig blood (see references 2 and 3).

Colon bacilli—electrophoretic migration velocities of less than 52 microns/100 volts/sec./3.45 cm. (see references 4 and 5).

Agglutination—titers of 1:160 and over.

There did not seem to be a relationship between the agglutination titer and the strength of the in vitro reaction (Figs 1, 2, and 3). Therefore, it will simplify comparison to consider only qualitative relationships between the in vitro tests and agglutinability (titers of 1:160 and over). These showed agreement in 72.5 per cent of *Staphylococcus albus* and in 67.4 per cent of colon bacilli, but other groups showed agreement in only about one half of the cultures (Table I).

Agglutinable cultures did not give stronger in vitro reactions than inagglutinable cultures. Certain cultures which gave strongly positive in vitro reactions were inagglutinable. Rawls and Chapman<sup>2</sup> found that, when a streptococcus which was resistant to fresh guinea pig blood was not agglutinated by the patient's serum, it had greater power to produce arthritis in rabbits than did similar but agglutinable strains.

### CONCLUSIONS

In vitro tests which had given results parallel with certain pathogenic properties were applied to cultures of staphylococci, streptococci, and colon bacilli isolated from persons suspected of having chronic infection. The cultures were also tested for agglutinability by the serum of the person from whom they had been isolated.

There was no close relationship between the in vitro reaction and the agglutination titer.

There was agreement in 67.4 per cent of colon bacilli and in 72.5 per cent of *Staphylococcus albus*, but there was agreement in only about one half of the cultures of streptococci and *Staphylococcus aureus*.

### REFERENCES

- 1 Chapman, G. H., Berens, C., Peters, A., and Curcio, L. Coagulase and Hemolysin Tests as Measures of the Pathogenicity of Staphylococci, *J. Bact.* 28: 343, 1934.
- 2 Rawls, W. B., and Chapman, G. H. Experimental Arthritis in Rabbits: Comparison of the Arthritis Producing Ability of the "Bactericidal" Action of Fresh Pig Blood and Those Which Are Agglutinable but Not Bactericidal, *J. Lab. & Clin. Med.* 21: 49, 1935.
- 3 Chapman, G. H., and Rawls, W. B. Studies of Streptococci. I. Quantitative Differences in Resistance to Various Agents, *J. Bact.* 32: 323, 1936.
- 4 Chapman, G. H., Stiles, M. H., and Berens, C. The Isolation and In Vitro Testing of Pathogenic Types of Non-Exotoxigenic Streptococci, *Am. J. Clin. Path. Tech. Suppl.* 9: 20, 1939.
- 5 Chapman, G. H. Electrophoretic Potential as an Aid in Identifying Strains of the B. coli Group, *J. Bact.* 18: 339, 1929.
- 6 Chapman, G. H., and Lieb, C. W. Certain Electrophoretic Relationships of Colon Bacilli, *J. Bact.* 37: 21, 1939.
- 7 Spicer, S. A Quick Method for Preparing Homogenous Suspensions of Hemolytic Streptococcus Antigens for Agglutination Testing, *J. Immunol.* 19: 445, 1930.
- 8 Spicer, S. A Rapid and Simplified Method for Conducting the Agglutinin Absorption Test of Hemolytic Streptococci, *J. Immunol.* 21: 141, 1931.
- 9 Mueller, J. H., and Khise, K. S. An Agglutinative Classification of the Hemolytic Streptococci of Scarlet Fever, *J. Infect. Dis.* 52: 139, 1933.
- 10 Steinberg, B., and Wiltzie, C. O. Skin Reactions to the Colon Bacillus and Its Toxic Products, *J. Immunol.* 22: 109, 1932.

# SODIUM DEHYDROCHOLATE SOLUTION AS A SOLVENT FOR NEOARSPHENAMINE IN THE TREATMENT OF SYPHILIS\*

CLARENCE SHAW, M.D., CHICAGO, ILL.

**I**N THE course of the treatment of syphilis with the trivalent arsenicals one occasionally finds patients who have a persistent gastrointestinal intolerance for these drugs. Although the number of such patients is small, they nevertheless present an important therapeutic problem because of the necessity of continuing arsenical treatment in some of them.

In general, there are two types of gastrointestinal reactions: immediate and delayed. The former type, occurring during or shortly after the administration of these drugs, may be due to a number of factors, such as gagging produced by the characteristic odor of these drugs, "speed" shock, colloidoclastic shock, "tubing" reaction, nitritoid reaction, or the patient having eaten just prior to receiving the drug. For the most part, simple well-known measures will alleviate these reactions.

The delayed type of gastrointestinal reaction, which occurs two to five or more hours after treatment, is not so easily prevented. Changing from one drug to another, although frequently successful, does not always solve the problem. These delayed reactions are thought to be due to the arsenicals temporarily interfering with normal liver function.<sup>1, 2</sup>

With this in mind, it has been suggested that using as a vehicle for the arsenical a preparation which increases bile flow, such as sodium dehydrocholate, would help prevent reactions due to biliary dysfunction. Appel<sup>2, 3</sup> demonstrated the value of this drug in the treatment of arsenical hepatitis and as a solvent for neoarsphenamine in patients who did not tolerate neoarsphenamine dissolved in distilled water. Savulescu<sup>4</sup> and Costinescu<sup>5</sup> have shown that neoarsphenamine dissolved in a 5 per cent sodium dehydrocholate solution is therapeutically active. It is thought that this combination of drugs results in a mixture rather than a new chemical compound.

In an effort to further evaluate sodium dehydrocholate, we selected a group of male patients who had been unable to tolerate one or all of the trivalent arsenicals because of gastrointestinal reactions. Only those patients who received at least five doses of neoarsphenamine dissolved in the sodium dehydrocholate solution have been selected. Because the group was small, we tried the use of the new vehicle in intolerant patients before changing to another drug. In all patients the technique of administration was identical with or without the sodium dehydrocholate. Care was taken to avoid undue aeration and to give all

\*From the Syphilis Division of the Public Health Institute, Chicago.  
Received for publication, November 16, 1938.

injections slowly. Patients selected were treated with gradually increasing doses of neoarsphenamine dissolved in a 5 per cent solution of sodium dehydrocholate (decholin sodium\*). Five cubic centimeters of the 5 per cent solution were used for every 0.15 gm. of neoarsphenamine.

In a group of five patients in whom nausea and vomiting occurred immediately after treatment, sodium dehydrocholate was ineffective in preventing these reactions. If the mixture did not succeed on the first trial, it was not tried again and an attempt was made to change to another arsenical.

TABLE I

INCIDENCE OF TOLERANCE OF NEOARSPHENAMINE DISSOLVED IN SODIUM DEHYDROCHOLATE SOLUTION

PATIENT'S NUMBER	NUMBER INJECTIONS WITH SODIUM DEHYDROCHOLATE	NUMBER WELL TOLERATED	NUMBER NOT TOLERATED	SUBSEQUENTLY TOLERATED MAPHARSEN
177333	10	10	-	Yes
173553	13	12	-	
170757	11	9	2	Yes
177494	11	1	-	
179103	8	8	-	
178684	8	7	1	Yes
16630	11	10	1	
173189	8	8	-	Yes
163874	10	10	-	
168783	5	5	-	Yes
166574	5	5	-	Yes
164745	6	6	-	
Total	110	103	7	6

In a group of twelve patients with the delayed type of gastrointestinal reaction to neoarsphenamine, the results were much better. Out of a total of 110 injections given, 103 were well tolerated, i.e., there was no nausea or vomiting. However, six of the twelve patients were later able to tolerate mapharsen with out gastrointestinal reactions. Table I illustrates the results in those patients in whom delayed nausea and vomiting occurred.

#### DISCUSSION

Five per cent sodium dehydrocholate solution as a solvent for neoarsphenamine was without value in those patients in whom nausea or vomiting occurred shortly after treatment, but in those patients in whom these reactions were delayed, it was capable of preventing reactions in 103 out of 110 trials. In two patients the drug was not permanently effective. After these patients had been satisfactorily carried on for eight doses with the mixture, they again became intolerant. No attempt was made to resume the use of sodium dehydrocholate after a rest in these two individuals. (Occasionally a patient would become nauseated some time during a course of neoarsphenamine dissolved in sodium dehydrocholate solution, but usually the next injection of the same mixture would be well tolerated.) In six patients the drug was discontinued after a reasonable trial—at least five doses—and mapharsen was used without reactions.

The added expense of using sodium dehydrocholate must be considered. However, in those cases where arsenicals are essential and where they can be used

\*Supplied through the courtesy of Riedel de Haen Inc.

only when dissolved in a solution of this or a similar drug, the difference in the cost can be compensated for by the greater therapeutic value of the arsenicals as compared with prolonged use of heavy metals.

### SUMMARY

In those patients who develop nausea and vomiting two to five hours after receiving an injection of neoarsphenamine, the reaction is thought to be due to the hepatotoxic character of the arsenicals.

Twelve such patients were given 110 doses of neoarsphenamine dissolved in a 5 per cent solution of sodium dehydrocholate (decholin sodium). Five cubic centimeters of sodium dehydrocholate solution were used for every 0.15 gm. of neoarsphenamine. One hundred and three of the 110 injections were well tolerated, i.e., no nausea or vomiting occurred. Six of the twelve patients were later able to tolerate a different drug (mapharsen). In two patients neoarsphenamine dissolved in sodium dehydrocholate solution was well tolerated at the beginning of the course but later there was the same reaction that occurred without the use of the sodium dehydrocholate.

Sodium dehydrocholate was valueless in preventing reactions in those patients in whom nausea and vomiting occurred immediately after treatment with neoarsphenamine.

Five per cent sodium dehydrocholate solution used as a solvent for neoarsphenamine widens the range of use of this drug in that it permits further use of this arsenical in those patients who develop the delayed type of gastrointestinal reaction.

The author is greatly indebted to his chief, Dr. J. E. Kemp, for valuable suggestions and permission to do this work.

### REFERENCES

1. Osborne, E. D., Putnam, E. D., and Hitchcock, B. S.: The Effect of Arsenic on Rabbits: Microchemical Studies Following the Administration of the Arsphenamines and Tryparsamide, *Arch. Dermat. & Syph.* 25: 419, 1932.
2. Appel, B.: Sodium Dehydrocholate in Arsphenamine Poisoning: A Preliminary Report, *Arch. Dermat. & Syph.* 27: 401, 1933.
3. Appel, B., and Jankelson, I. R.: Treatment of Arsenical Hepatitis With Sodium Dehydrocholate: Experimental and Clinical Studies in Cases of Arsphenamine Poisoning, *Arch. Dermat. & Syph.* 32: 422, 1935.
4. Savulescu, A.: Die Behandlung der Syphilis mit in gallensauren Salzen gelösten Arsenobenzolen, *Soc. med. d. hop. de Bucharest* 12: 220, 1930.
5. Costinescu, C.: A New Method for the Treatment of Syphilis in Which Large Doses of Arsphenamine Dissolved in Bile Salts Are Used, Bucharest, 1930, Unpublished Thesis.

# LABORATORY METHODS

## INTRADERMAL TEST AS AN AID IN THE DIAGNOSIS OF ENTEROBIASIS\*

H TSUCHIDA, Sc D, AND T C BAUFELTIN, M D, St Louis, Mo

DESPITE the common occurrence of enterobiasis its clinical diagnosis is frequently open to errors chiefly due to difficulties in differentiating pruritis accompanying this infection from that attributable to other disturbances. The detection of ova of *Enterobius vermicularis* in the stool is likewise confronted with failure. Thus, according to Craig and Faust,<sup>1</sup> the ova of the parasite were found only in less than 5 per cent of infected individuals. In order to facilitate the diagnosis, therefore, additional methods have been suggested, namely, by the finding of the ova in perianal swabs such as recently introduced by Hall<sup>2</sup> or from under the fingernails, or by the recovery of adult worms by means of enemas. Though these methods are apparently superior to fecal examination, lack of proper cooperation on the part of patients may become a source of error in the diagnosis.

*Enterobius vermicularis* is generally considered as a strictly lumen parasite of the intestinal tract. The clinical pictures due to the presence of the parasites are at times not clear cut. However, catarrhal inflammation of the immediate tissues caused by undue attachment of the worms may induce the absorption of toxins secreted by the worms. This may lead to nervous and systemic disturbances often observed in the patients. Schropl<sup>3</sup> was of the opinion that the parasite secretes a toxin and that the frequency of the parasite and its connection with obscure common complaints should always be investigated. For this reason, the present study was undertaken to determine the relative value of the intradermal test as an aid of establishing the diagnosis of enterobiasis.

### METHODS AND OBSERVATIONS

Grubel<sup>4</sup> was the first to introduce the intradermal test in the diagnosis of enterobiasis. The antigen prepared by him consisted of a saline emulsion of *Enterobius vermicularis*. He demonstrated that an immediate reaction developed in infected individuals within five minutes, with a progressive itching accompanied by a rash (Quaddelbildung). He also found that the extract was reactive after heating to boiling point. Schropl later, by using the alcoholic extract of the worms, reported that a delayed reaction was obtained in 13 out of 15 infected

\*From the Departments of Bacteriology, Immunology and Public Health and of Internal Medicine respectively, Washington University, School of Medicine.  
Received for publication May 31 1938

individuals, while individuals with both negative history and stool gave no reactions. Recently, Wright and Bozicevich<sup>2</sup> compared the value of dermal and intradermal tests by the use of physiologic or buffer saline extracts of the worms, and claimed the higher specificity for the dermal test.

*Preparation of the Antigen.*—A large number of adult worms were recovered from the stool of a patient following purgation with a dose of magnesium citrate. The worms were carefully washed one by one by first dipping into several changes of sterile normal saline solution, and finally washing them down rather forcibly with the solution. The worms were then placed in a sterile Petri dish and left standing in an incubator at 37° C. until dried. These were further placed either in a vacuum over sulfuric acid or treated by freezing with lyophile apparatus until thoroughly dried, after which they were pulverized. The latter was extracted with absolute alcohol at room temperature for two days. The residue after evaporation was completely dried and then extracted for forty-eight to seventy-two hours with Coea solution (sodium chloride 0.7 per cent, sodium bicarbonate 0.05 per cent, and phenol 0.33 per cent in distilled water). After centrifuging, the supernatant fluid was drawn off, filtered through a Seitz filter, and stored in ampoules or vials in the refrigerator.

#### SKIN REACTION IN EXPERIMENTAL ANIMALS

With the use of the antigen thus prepared, three rabbits (one naturally and two experimentally infected with *Passalurus ambiguus*) were tested. The tests were made on the abdomen of the animal with Coea solution as a control. The results showed that the naturally infected animal gave uniformly positive reactions of more or less delayed type with 0.1 c.c. of 1:100, 1:500, and 1:1000 dilutions of the antigen, but not with 1:2000 dilution. The reactions developed one hour after the injection and persisted usually for twenty-four hours, at which time, however, erythema completely disappeared, leaving only a slightly edematous area. Control animals which were tested invariably gave negative reactions. The two experimentally infected animals showed the initial skin response on the thirty-first and thirty-fourth days, respectively, following the oral administration of washed ova of the parasites. One of the animals was later treated twice with hexylresorcinol. When no ova were recovered from the fecal pellets upon repeated examinations, the animal was removed to a new cage in order to prevent a possible re-infection. This animal was subsequently given by stomach tube 2000 trichinella larvae isolated from infected muscle by artificial digestion. The skin test with the enterobius antigen became negative three months later, while the test with trichinella antigen (1:1000) prepared by McCoy, Miller, and Friedlander<sup>3</sup> gave a definitely positive reaction. This result seemed to indicate that no group reaction occurred in the animal with trichinella infection by the use of the enterobius antigen. All the rabbits also harbored oocysts of *Eimeria stiedae* which indicated that the reaction was apparently specific for *Passalurus ambiguus*. In order to further determine the specificity of the antigen, 8 guinea pigs were experimentally infected with trichinella larvae. After an elapse of three weeks, the animals were tested with the enterobius antigen and found to be negative in all instances. In the fecal pellets of these animals were intestinal protozoa including balantidia.

## SKIN REACTIONS IN MAN

Patients with the complaints of pruritis ani were tested with 0.05 cc of 1:500 dilution of the enterobius antigen on the flexor surface of the forearm. A control was run with Coea solution to eliminate certain type of pseudo positives. If the reaction was positive, differential blood counts were made, emphasis being placed on the percentage of eosinophilic leucocytes. They were requested to submit to us a complete bowel movement following purgation with magnesium citrate or a copious enema. Careful search for adult worms was then instituted. This was done by floating out in clear water the material caught in the sieve (40 meshes per inch) and searching for the worms in a thin layer in the flat bottom of Petri dish over a dark background.

The positive skin reactions obtained are of immediate type and usually begin to develop in from five to ten minutes. They are characterized by the formation of a wheal of varying size with irregular pseudopodial margin surrounded by a zone of erythema. The reactions are usually read after twenty minutes. No test is considered positive in which there is not a definite wheal at least 7 mm in diameter and a zone of erythema not less than 20 mm in diameter. Definite positive reactions are quite clear with a wheal 10 to 20 mm in diameter surrounded by an erythema up to 50 mm, with or without itching. Tentatively an erythema of 25 to 30 mm in diameter is designated as + reaction, 35 mm, ++, 40 mm, +++, and 50 mm or over, ++++ reactions. The reaction as a rule, disappears within an hour or two. A slight brown discoloration of skin at the site of injection has often been observed by one of us (T. C. B.). Whether it represents dermatropic necrotizing effect of the toxins liberated by the worms is difficult to determine at this time.

The results of the study showed that 10 control persons who have had neither clinical symptoms nor the presence of the parasite in the stools showed negative skin reactions. Five persons with pruritis ani gave positive reactions, but no parasites were found upon repeated examinations. This may be explained by the fact that some individuals are hypersensitive to any proteins injected into the skin, or probably the reaction may be due to the comparative low dilution of the antigen used. Augustine and Theiler, in their work on trichinosis cited instances in which the number of positive reactions decreased when high dilutions of the antigen were used.

Nineteen cases with complaints of pruritis ani and the presence of the adult worms in the postcothartie stools showed positive reactions within twenty minutes following the injection of the antigen. In none of these cases were the number of eosinophiles over 2 per cent, and in one instance no eosinophiles were present. This seemed to indicate that no apparent positive correlation existed between the number of eosinophiles and enterobius infection. Panlian<sup>5</sup> claimed the presence of eosinophilotoxic substances in the blood of infected individuals. These substances were found by him to be alcohol and water soluble. One of the cases under observation gave a negative reaction with a positive stool at the first visit but developed a strongly positive reaction two weeks later, indicating that the skin response to the enterobius antigen did not develop in the early stage of the infection.



Our observations lead us to believe that those who have had the infection and have been cured are apt to show a slightly positive reaction over a long period of time. It is for this reason that at least a two-plus reaction only should be considered diagnostic. The reaction, as a rule, begins to fade quite promptly after recovery, maintaining, however, a very low grade of response for some time to come.

#### COMMENT

The mechanism of the intradermal test in this and other helminthic infections is little understood. The test depends either upon hypersensitivity to certain materials contained in the antigen or upon injury of normal tissue by a definite toxin in the absence of specific antibody. It is highly probable that in individuals harboring pathogenic parasites, elaboration of antibodies has sensitized the skin. As a result, the introduction of a minimal amount of antigen derived from the homologous, or a closely related species, gives rise to an immediate local response. That the group specificity apparently exists in the case of *Enterobius vermicularis* was well demonstrated in the instances of rabbits infected with *Passalurus ambiguus*.

Judging from the results obtained in this study, we may say that the intradermal test is probably of value in diagnosing cases of enterobiasis in which pruritis ani is a predominant symptom (19 out of 24). Five cases with pruritis ani which gave positive skin reactions and negative stools, may be referable to disturbances other than of helminthic origin, such as neurologic or allergic conditions, or else fungoidal etiology may be sought for in this connection. In control persons, the test was specific in that none responded. Though the number of normal individuals tested in this series was too few to justify any conclusion, the test appeared to be useful in eliminating the possibility of enterobiasis.

From the practical point of view, the intradermal test in enterobiasis cannot be very well considered a certain diagnostic method. It should always be checked with parasitologic proof obtained by the methods previously described. The diagnosis, in our opinion, may be best made by searching for the adult worms in the postcathartic stool, or one following a copious enema.

#### SUMMARY

1. Experimental study made with enterobius antigen showed that 3 rabbits infected with *Passalurus ambiguus* gave definitely positive reactions with 1:100, 1:500, and 1:1000 dilutions, and not with 1:2000 dilution of the antigen. This illustrates an instance of group-specific reactions. One of the animals which was later treated and apparently recovered from the infection gave negative reaction along with the control animals. Furthermore, one rabbit and 8 guinea pigs infected with trichinella larvae did not respond to the enterobius antigen, indicating the specificity of the reaction.

2. Thirty-four individuals were tested with the enterobius antigen. Ten control persons gave negative reactions. Of 24 persons with complaints of pruritis ani, 19 developed positive reactions and the worms were found in the stools, while five gave positive reactions and negative stools. In one case of enterobiasis no reaction was elicited until two weeks after the recovery of the adult worms in postcathartic stools. This seemed to suggest that the tissue response to the enterobius antigen was lacking in the early stage of the infection.

3 Negative tests were apparently more valuable in indicating evidence of freedom from enterobiasis than were the positive ones in establishing it. However, the test should always be checked with parasitologic examination.

#### REFERENCES

- 1 Craig, C F, and Faust, E C. *Clinical Parasitology*, Philadelphia, 1937, Lea & Febiger
- 2 Hall, M C. *Studies on Oxyuriasis. I Types of Anal Swabs and Scrapers, With a Description of an Improved Type of Swab*. *Am J Trop Med* 17: 440, 1937
- 3 Schrepl, L. *Hautproben mit Oxyurenextrakt*, *Deutsche med Wchnschr* 52: 1508, 1926
- 4 Grubel, E. *Spezifische Hautreaktion bei Infungen mit Extraktion von menschlichen Parasiten (Oxyuren, Lause, usw.)*, *Dermat Wchnschr* 79: 1182, 1924
- 5 Wright, W H, and Bozicevich, J. *Studies on Oxyuriasis. VI Dermal and Intradermal Skin Reactions in Oxyuriasis*, *J Parasitol* 23: 562, 1937
- 6 McCoy, O R, Miller, J I, and Friedlander, R D. *The Use of Intradermal Test in the Diagnosis of Trichinosis*, *J Immunol* 24: 1, 1933
- 7 Augustine, D L, and Theiler, H. *Precipitin and Skin Tests as Aids in Diagnosing Trichinosis*, *Parasitology* 24: 60, 1932
- 8 Paulian, D E. *Sur les lésions des Vers Intestinaux*, *Presse mcd* 23: 403, 1915

### A NEW INSTRUMENT FOR AUTOMATICALLY RECORDING THE ERYTHROCYTE SEDIMENTATION RATE AND THE VOLUME PERCENTAGE OF CELLS AND PLASMA UPON A SINGLE PERMANENT RECORD

ROY D. NICHOLS, B V Sc, M S, COLUMBUS, OHIO

**A**FTER a thorough review of the subject of sedimentation of blood cells, it was realized that of the many methods of recording the phenomenon, none accurately measured all phases of the rate of descent of the line of demarcation between cells and plasma. One method,<sup>1</sup> based upon the fact that light passes through plasma and not through cells, suggested the possibilities of photography as a means of automatically recording the rate.

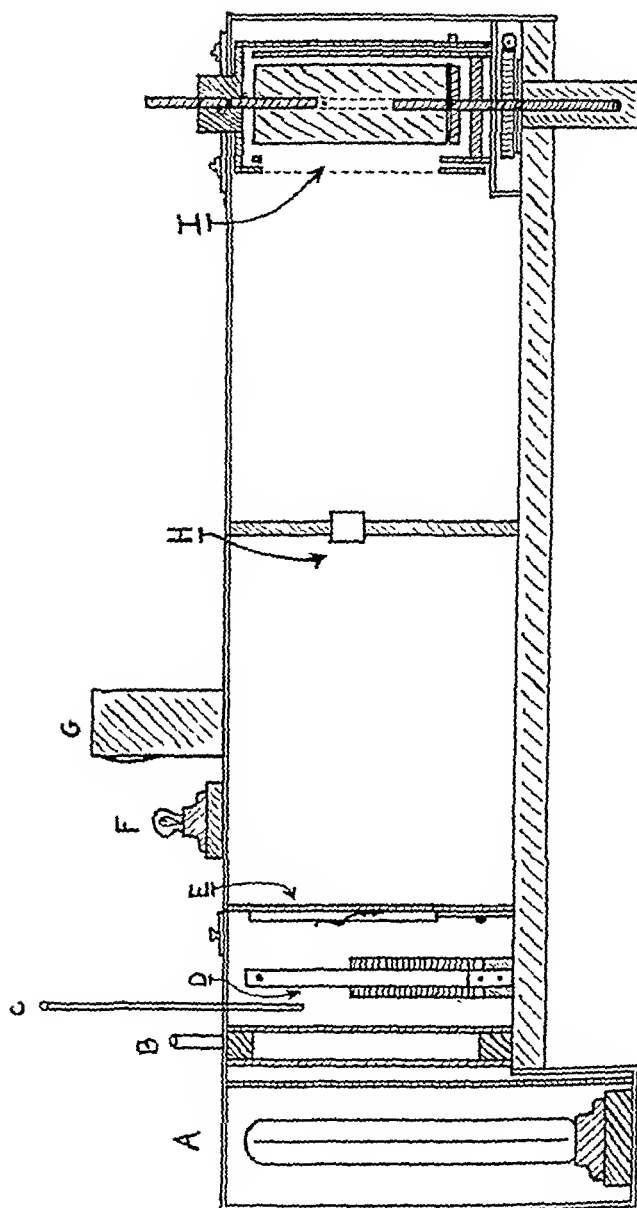
With the cooperation of F W Davis, of the Photography Department of Ohio State University, various photographic methods were tried, until finally it was decided to attempt to build an instrument that would transfer an image of a very narrow vertical portion of a blood filled tube through a photographic lens onto sensitized paper attached to a revolving drum.

Fig 1 illustrates the mechanics as applied to this principle.

A represents the light source, a 75 watt tubular showase bulb with a long filament to illuminate a Wintrobe hematocrit tube<sup>2</sup> fairly uniformly from top to bottom.

B is a glass walled chamber or condenser through which water is run to eliminate the heat of the light source and cool the chamber containing the blood sample to well below room temperature. There is a pane of glass between A and B establishing an air chamber between the bulb housing and water jacket. This further aids in eliminating the heat of the light source.

<sup>1</sup>From the College of Veterinary Medicine Ohio State University, Columbus  
Received for publication May 31 1938



*C* is a thermometer inserted in the chamber containing the blood sample.

*D* represents a thermostatically-controlled nonglowing heating element incorporated to maintain in the chamber containing the blood tube any temperature that may be desired above that produced by the cooling system. At present the thermostats are regulated to hold the temperature at approximately  $37^{\circ}$  C. while the apparatus is in use. The heating element and thermostat are so constructed as not to interfere with the proper illumination of the Wintrobe tube.

*E* is a blood tube holder containing a vertical slit about 0.003 of an inch wide. This holder is perpendicular to the flat table top upon which it is built. With the aid of water levels and adjustable screws in the legs, the table is leveled.

To minimize vibration, the table is placed on a cement floor. To further and in minimizing vibration, the motor which activates the drum is mounted upon rubber and also fastened to the cement floor.

*T* is a red pilot light for assistance in adjusting the thermostat.

*G* is an electric time switch clock.

*H* represents a photographic lens which transfers in proper focus an image of a very narrow portion of the Wintrobe tube onto photographic paper on drum *I*.

*I* is a drum for holding sensitized paper. This drum is perpendicular to the flat table top and made to travel at a predetermined slow speed by means of a motor and reducing gears. The speed of the drum may be altered if desired, but at present it is 0.92 r.p.h. This speed allows for a recording of sedimentation for one hour with additional space left on the sensitized paper to photograph the sample after centrifugation. Around the drum is a housing and shutter. These are so constructed that the sensitized paper may be placed upon the drum in a darkroom. The portion of the apparatus containing the sensitized paper (drum, housing, and shutter) may then be transferred to the unit in daylight and the shutter opened from outside of the unit. The apparatus from the blood tube holder to and including the drum is light tight and the remaining anterior portion can be made so at will if the instrument is to be used in a dark room.

Three such units have been built and have produced very gratifying results. Although designed to more accurately establish "normals and physiological variations" in the blood of equines, the apparatus is sufficiently flexible to make its use possible with human blood or that of other animals.

Fig. 2 illustrates the results obtained from a half hour exposure of horse's blood and a one minute exposure of the same sample after centrifugation.

*DD'* is the top of the dark heavy line and represents the bottom of the column of blood. The length of *DD'* (or *AA'*) represents the time of exposure.

*AD* represents the height of the column of blood (100 mm.) at the start of the measurement when the cells are dispersed throughout the plasma.

The dark area *ABCA'* represents the exposed area of the graph brought about by the increasing amount of light passing through the plasma as the cells settle out.

The light area *ABCD'D* represents the unexposed area brought about by the presence of cells through which the light cannot pass.

*AB* represents the beginning and gradually accelerated descent of the line of demarcation between cells and plasma.

*BC* represents the gradually retarded descent of the line of demarcation between the cells and plasma brought about by the gradually increasing effect of heaping of cells in the lower portion of the tube.

*B* represents the theoretical point of change between the gradually accelerated descent (*AB*) and the gradually retarded descent (*BC*) of the line of demarcation between cells and plasma.

Sigmoid curve *ABC* then represents a graphic record of the descent of the line of demarcation between cells and plasma for a given time.

The small light horizontal lines in area  $ABCA'$  represent the millimeter divisions on the tube.

The small vertical lines in area  $ABCA'$  occur at intervals of approximately 1.0174 minutes as the apparatus is now run.

The narrow dark vertical lines preceding and following exposure of the sedimenting and centrifuged sample are made by slight exposure of the sensitized paper when the tube holder is empty.

$UW$  represents the height of the column of blood (100 mm.).

$UV$  represents the height of the column of plasma after centrifugation (volume percentage of plasma).

$VW$  represents the height of the column of cells after centrifugation (volume percentage of cells).

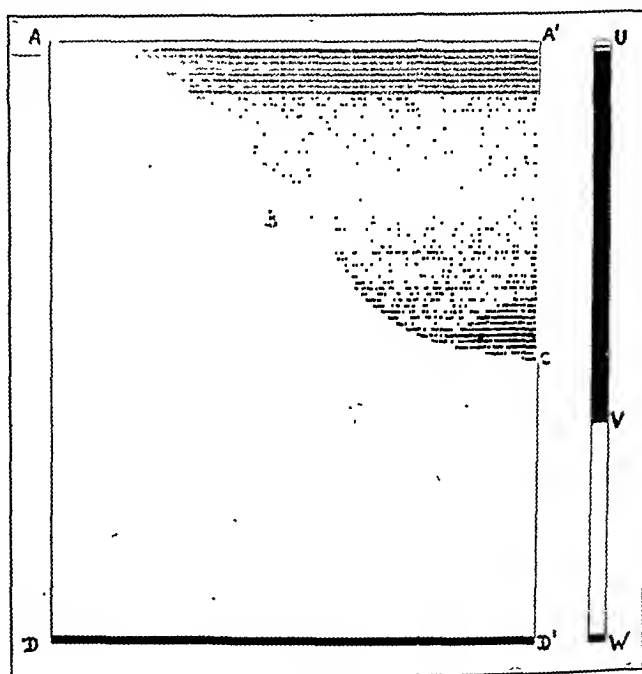


Fig. 2.

This mechanical means of measuring and recording cellular sedimentation is thought to embody the following advantages over previous methods:

1. It measures all phases of the sedimentation phenomenon as it occurs in the Wintrobe hematocrit tube.

2. It measures the rate of sedimentation continuously and automatically.

3. The record is free from personal error in measuring.

4. The record is permanent.

5. Volume percentages of cells and plasma for the same sample of blood are permanently incorporated upon the same record as the sedimentation rate. If desired one may alter the ratio of formed elements and plasma to a previously decided upon standard volume and photograph the corrected sedimentation rate and volume percentages of cells and plasma for comparison with the original sample.

6 Time heretofore consumed in periodically reading the position of the line of demarcation between cells and plasma can be used for other useful laboratory examinations

7 More than one unit facilitates the comparison of more than one sample at the same time This fact is a great asset in problems similar to the one now being conducted involving "standardization" of technique and establishment of "normals and physiological variations"

8 If one desires an accurate permanent record of the standardization of a centrifuge as to speed and time necessary to completely pack the formed elements of blood in the Wintrobe hematocrit tube, one needs only to stop the centrifuge at intervals and photograph the tube in the unit, repeating the procedure until no further packing occurs

9 To standardize a shaking device for the proper agitation of blood samples prior to sedimentation determinations, the apparatus may be used for accurate permanent recordings

10 It takes little thought to find uses for the apparatus in many phases of laboratory work other than in the field of hematology

The apparatus described was manufactured for the author by W F Sipes of Columbus Ohio

#### REFERENCES

- 1 Sulikowitch, H W A Photographic Suspension Stability Apparatus, Am J M Sc 187 C7, 1934
- 2 Wintrobe, W W A Simple and Accurate Hematocrit, J LAB & CLIN MED 15 287 1930

## THE USE OF AIR COOLED CONDENSERS IN BLOOD CHOLESTEROL DETERMINATIONS\*

I BREH, PH D, DETROIT, MICH

IN THE determination of blood cholesterol by methods employing the Liebermann-Burchard reaction, contamination of the chloroform extract of the blood with water should be carefully avoided, otherwise erroneous values will be obtained In many clinical laboratories Lieboff tubes and water cooled condensers are used in preparing the chloroform extract Recently, some rather expensive modifications of this apparatus have been put on the market which permit moisture that forms on the outside of the condenser to trickle into the tube and thus to interfere with proper extraction or with subsequent color development This usually occurs only when the tap water is very cold, or when the relative humidity in the laboratory is high The difficulty is readily overcome by using air cooled condensers, the cost of which is also negligible Pyrex glass tubing, about 30 inches long and one half inch outside diameter, will serve the purpose The end, which passes through the cork fitting into the Lieboff tube, is drawn out somewhat From the standpoint of simplicity and economy, as well as reliability, this arrangement leaves little to be desired

\*From the Department of Laboratories Henry Ford Hospital Detroit  
Received for publication May 16 1938

## DETERMINATION OF HIPPURIC ACID IN URINE<sup>\*</sup>

T. E. WEICHELBAUM, PH.D., AND J. G. PROBSTEN, M.D., ST. LOUIS, MO.

QUICK some time ago recommended the estimation of hippuric acid that is excreted in the urine after the ingestion of benzoic acid as a test for liver function.<sup>1</sup> The test has two merits: it is simple and it gauges a well-defined function of the liver, i.e., its ability to conjugate benzoic acid with glycine. When the method came into use in this laboratory for various studies, the analytical technique described by Quick proved to be unsatisfactory, as it yielded inaccurate and erratic results. Quick determines either gravimetrically or volumetrically the amount of hippuric acid which crystallizes out upon acidification of the urine. Aware of the fact that the substance does not separate out completely, Quick adds to the amount of the hippuric acid found 0.33 gm. for each 100 c.c. of mother liquor. This method of correction implies the assumption that 100 c.c. of urine retain at ordinary temperatures 0.33 gm. of hippuric acid in solution and that all in excess of this amount crystallizes out.

Our experiments did not bear out this assumption. We found, namely, that the solubility of hippuric acid in urine at 22° to 26° C. is in the first place considerably greater than 0.33 per cent, and in the second place that it shows substantial variations with different urine specimens. In the experiments which disclosed this fact, known quantities of pure hippuric acid were dissolved in measured volumes of urine samples and subsequently recovered with strict observation of the directions given by Quick. As shown in Table I, the solubility of hippuric acid was in every instance much greater than 0.33 per cent; the lowest figure we found was 0.44, the highest 0.62 gm. per 100 c.c. of urine. To use for correction the average of values which spread over such a wide range would be too crude a procedure. The high degree of solubility of hippuric acid, furthermore, greatly limits the usefulness of the method, since if a urine contains no more than 0.5 to 0.6 gm. of the substance per 100 c.c., none of it separates out after acidification, and the urine passes analysis as containing no hippuric acid at all; likewise, if the hippuric acid content is 0.25 to 0.30 gm. per 100 c.c., it eludes detection even if the urine is concentrated to one-half of its original volume.

In view of the merits of the test we attempted to increase the accuracy of the analytical technique. Our purpose was substantially to diminish, and at the same time to render more uniform, the solubility of hippuric acid in various

<sup>\*</sup>From the Laboratory of the Jewish Hospital of St. Louis. This work aided by the David May-Florence G. May Fund.

Received for publication, June 2, 1935.

TABLE I  
SOLUBILITY OF HIPPURIC ACID IN URINE

GRAMS HIPPURIC ACID ADDED	VOLUME URINE CC	GRAMS HIPPURIC ACID RECOVERED	GRAMS HIPPURIC ACID HELD IN SOLUTION PER 100 CC URINE
0.5	50	0.211	0.378
1.0	50	0.720	0.560
1.0	50	0.687	0.626
1.0	100	0.506	0.494
1.0	100	0.594	0.606
2.0	100	1.510	0.440
1.0	150*	0.759	0.482
1.0	150*	0.777	0.526

\*Evaporated to 50 cc before analysis

samples of urine, without making the method unduly tedious for use in clinical diagnostic work. A simple step, i.e., saturation of the urine with sodium chloride, produced the desired conditions. As the figures in Table II show at ordinary room temperatures 100 cc of urine in which 30 gm of sodium chloride is dissolved, holds in solution only 0.110 to 0.137 gm of hippuric acid. Thus its solubility has been cut to nearly one fifth and the variations have become relatively slight, the difference between extreme figures has been reduced from (626-440) 186 to (137-110) 27 mg per 100 cc. Hence the average of the solubility figures, 0.123 gm per 100 cc of urine can be adequately used as a correction by addition to the amount of hippuric acid which had separated out from the acidified urine. This modification allows the analysis of 150 cc and even 200 cc of urine without preliminary concentration, whereas the maximum volume permitted by Quick is 100 cc. In addition the time required for the complete crystallization and separation of hippuric acid also is considerably shortened.

TABLE II  
SOLUBILITY OF HIPPURIC ACID IN URINE AFTER SATURATION WITH SODIUM CHLORIDE

GRAMS HIPPURIC ACID ADDED	VOLUME URINE CC	GRAMS NaCl ADDED	GRAMS HIPPURIC ACID RECOVERED	GRAMS HIPPURIC ACID HELD IN SOLUTION PER 100 CC URINE
0.5	100	0	0.77	0.12
0.5	100	20	0.770	0.10
1.0	50	15	0.920	0.120
1.0	100	28	0.880	0.120
3.0	100	0	0.870	0.10
1.0	100	0	0.890	0.110
1.0	100	20	0.884	0.114
1.0	150	45	0.816	0.12
1.0	150	45	0.795	0.17
			Average	0.123

The analytical procedure is carried out as follows. Measure the volume of the urine specimen. If it is above 150 cc, add a few drops of glacial acetic acid and evaporate to a volume not in excess of 150 cc. Add 30 gm of sodium chloride per 100 cc of urine and heat with shaking until the salt is dissolved. Cool the solution to about 15°-20° C by immersion of the flask into ice cold water, add 12 cc of approximately 10 N sulfuric acid and scratch the sides



of the flask with a glass rod to enhance the crystallization of the hippuric acid. (This apparently trivial step is important.) Allow to stand for fifteen minutes in the cold water bath, then filter through a Hirsch funnel (diameter of perforated plate 47 mm.), using moderate suction. Wash the precipitate with chilled 30 per cent sodium chloride solution from a wash bottle, using the washing fluid first to rinse the flask in which the precipitation has been performed. It is not necessary, however, to transfer the precipitate to the filter quantitatively. The precipitate is adequately washed when the washing fluid is free of sulfuric acid. Transfer the funnel with its contents onto the flask that still contains some of the hippuric acid crystals, and rinse the filtered hippuric acid into it by dissolving it in hot water; to this end use a fine tipped wash bottle. All of the hippuric acid is now in the flask in which it had been precipitated; heat until all the hippuric acid particles adhering to the flask dissolve, and titrate while hot with 0.5 N sodium hydroxide, with phenolphthalein as an indicator.

#### CALCULATION

The number of cubic centimeters of 0.5 N sodium hydroxide used for neutralization is multiplied by 0.072, which gives the amount of sodium benzoate in grams from which the hippuric acid was derived. If to this amount one adds the correction for the solubility of hippuric acid (0.123 gm. per 100 c.c. of urine) in terms of sodium benzoate, i.e., 0.123 by  $0.804 = 0.10$  gm. sodium benzoate, one obtains the sodium benzoate value of the specimen with a maximum error of  $\pm 10.5$  mg. per 100 c.c. *Example:* The volume of the urine specimen analyzed (excreted during one hour after the ingestion of 4 gm. of sodium benzoate) was 134 c.c. The amount of sodium chloride added was 40 gm. (more accurately  $1.34$  by  $30 = 40.2$  gm.). The precipitated hippuric acid required 11.2 c.c. of 0.5 N sodium hydroxide for its neutralization. This corresponds to  $11.2$  by  $0.072 = 0.806$  gm. sodium benzoate. The correction for solubility would be  $1.34$  by  $0.10 = 0.134$  gm. sodium benzoate. The sodium benzoate value of the specimen equals 0.940 gm. The probable error is  $\pm 10.5 \times 1.34 = \pm 14$  mg. sodium benzoate, which corresponds to 1.4 per cent of the quantity determined.

Expressing the amount of the hippuric acid excreted in terms of sodium benzoate simplifies the visualization of its relationship to the amount of the sodium benzoate ingested. Thus our example shows that in one hour after the ingestion of 4 gm. of benzoate, 0.937 gm., or 23.4 per cent, of it had been excreted.

Occasionally one encounters urine specimens that are markedly bile-tinged or dark-colored. The analysis of such urines is difficult because the hippuric acid clogs the filters due to the admixture of colloids. A further difficulty in such cases is caused by the fact that the final hippuric acid solution is too highly colored and as a consequence the end point of the titration is difficult to determine. This difficulty can be easily overcome by the following procedure: Add 0.3 gm. of acid-washed norit per 100 c.c. of urine, and

boil the mixture for about one minute. After cooling filter by suction on a Hirsch funnel, and wash the nonit residue with a small quantity of hot water, measure the volume and proceed as outlined.

If the urine samples contain protein, this must be completely removed before the determination of the hippuric acid content. To this end, measure the volume of the sample, add 5 c.c. of 20 per cent copper sulfate solution, mix, then with agitation introduce 5 c.c. of  $N/1$  sodium hydroxide per 100 c.c. of urine. In exceptional cases with very large albumin content, double these amounts. Shake and heat to near the boiling temperature. After cooling filter through a good grade of filter paper into a graduated cylinder and record the volume of the clear filtrate. For the rest the procedure is the same as for normal urines, but for the fact that the urine was diluted with the protein precipitants and that only an aliquot portion was used for analysis. This must be taken into account in the calculation of the results.

#### SUMMARY

The analytical technique of Quick for the determination of hippuric acid in urine is inaccurate. A modification is proposed which renders the method sufficiently accurate for clinical diagnostic work while retaining its original simplicity. Directions are described for the preliminary treatment of urines which contain bile, dark coloring matter, or albumin.

#### REFERENCE

Quick, A. T. Clinical Value of Test for Hippuric Acid in Cases of Disease of Liver. *Arch. Int. Med.* 57: 544, 1936.

## A DIFFERENTIAL MANOMETER METHOD FOR THE MEASUREMENT OF BLOOD FLOW\*

HAMIRAN LAWSON, M.D., PH.D., AND J. P. HOLT, M.D. LOUISVILLE, KY.

THE principle of the Venturi meter was used in 1926 by Daly, and with modifications in 1927 by Wagoner and Livingston, for the measurement of blood flow. In neither case did the differential manometer employed permit a direct graphic recording of flow.

In order to obtain such a record without resorting to optical methods, a differential manometer was constructed which would permit the taking of direct mechanical records on smoked paper. In principle the manometer differs very little from the familiar Pachon capsule except that the membrane between the pressure chambers is double, which permits sealing each chamber airtight. Each chamber thus becomes a simple tambour. When the membranes have been attached (condom rubber) the chambers are bolted to leather, membrane to membrane, with the recording lever sandwiched between,

\*From the Department of Physiology and Pharmacology, University of Louisville School of Medicine.

Received for publication June 6, 1938.

cemented to one or both membranes with rubber cement. The lever passes out of the capsule through a shallow notch cut in the rim of both tambours (Fig. 1). In use, both chambers and all lead tubes are completely filled with 5 per cent sodium citrate solution.

A defect in all rubber membrane manometers to which attention has frequently been directed is the inconstancy of the membrane. This defect is present in a differential manometer of the type described, where the restoring force is the elasticity of the rubber membrane. We have observed slight changes in the calibration curve of these manometers for the first half hour or so following their completion, which we attribute in part to drying of the rubber cement, and in part to changes in the elasticity of the membrane. If the tambours are allowed to dry thoroughly, however, after the membranes have been applied, and two or three maximum deflections in both directions are produced, the manometer gives constant deflections thereafter for a period of one or two days, in rubber tube hydraulic systems. Since replacement of the membranes requires only a few minutes, it has been our practice to start each day's work with fresh membranes, allowed to dry for at least a half hour, and maximally stretched two or three times.

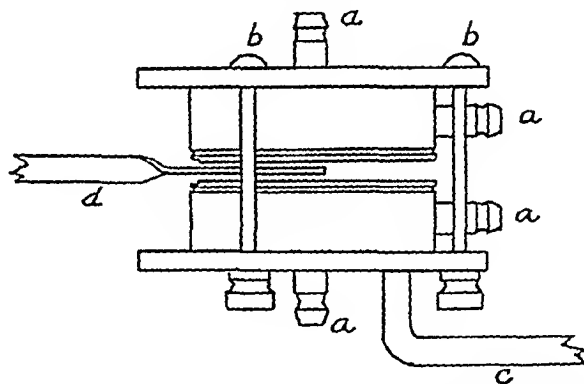


Fig. 1.—Semidiagrammatic sketch to show construction of membrane manometer. The two halves are slightly drawn apart to show insertion of the lever *d* between the membranes. The lever is a thin strip of phosphorbronze, about 3 mm. wide, which passes through a shallow notch cut in the rim of each chamber, to avoid binding. The membrane (not shown in the sketch) passes over this notch and is tied in its groove on each chamber. The bolts *b* are loosened to replace the membranes, and tightened when the manometer is in use. The apposed rims of the two chambers are made smooth and broad to avoid cutting the membranes when the bolts are tightened, except where the lever passes out of the capsule. Here the notched rim of each tambour is filed to a dull knife edge. Three bolts suffice, equally spaced about the circumference. Each chamber has two metal nipples *a* for connecting rubber tubing, to facilitate washing through with citrate solution. To wash out air bubbles in the lower chamber, the manometer is rotated about the supporting rod *c* until the wash-out tube in the lower chamber is uppermost. The inside diameter of each chamber is 3 cm., the inside depth 1.2 cm.

In order to apply the Venturi principle without using anticoagulants, Wagoner and Livingston constricted the aorta with an oblique ligature at the origin of a sizable branch, which was cannulated and led to one limb of the manometer. The other limb of the manometer was connected by cannulation with an artery originating above the constriction. We found it very difficult to place a dependable ligature so as to produce this type of constriction. In trials on animals and on rubber tube models, the pressure difference for a given flow was found to be at least as great if the lower limb of the manometer was connected below the constriction rather than in it. This procedure was, therefore, followed in all subsequent work.

Meissman (1916) gives the fall in head below a constriction in a tube as

$$H = \left( \frac{d}{d_1} - 1 \right)^2 \frac{v^2}{2g}, \text{ where}$$

$H$  is the loss of head,  $a$  the cross sectional area of the tube,  $a_1$  the area of the constriction, and  $v$  the velocity in the tube. Thus, with a constant constriction, the fall in head below the constriction is a function of the velocity of flow. No attempt was made in this work to produce a standard constriction in the artery. A small Gaskell clamp, with one limb cut through so that it could be swiveled into place on the vessel, was tightened down until pressure on the distal side fell 3.5 mm Hg. This was sufficient difference in pressure to give about half of the full deflection from the zero point of the manometer. The constriction, as well as the manometer, was calibrated at the end of each experiment, when absolute values for flow were required, by opening the

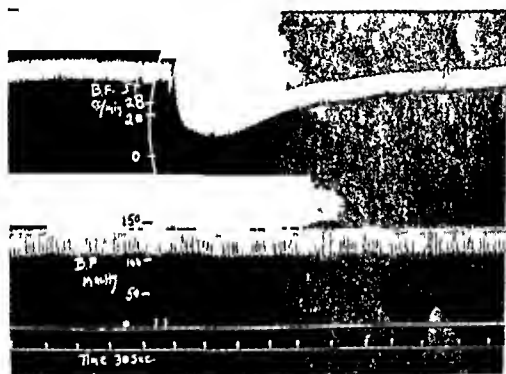


Fig. 2.—Records from above downward (1) blood flow into hind limb of bromitalized dog (2) carotid blood pressure (mercury manometer) (3) signal and base line for carotid pressure (4) time in thirty second intervals. Between signals 1 c.c. epinephrine 1:50,000 was injected into the femoral artery through the central stump of the ligatured internal iliac artery. Note the immediate transitory reduction in blood flow during the injection due to the rise in pressure in the femoral artery from the volume of fluid injected. This is followed by the typical constrictor effect of epinephrine.

artery below the lower limb of the manometer and measuring directly the flow required to produce manometer deflections observed in the experiment. As a rule, each deflection was checked against flow at least twice. In the second calibration blood pressure was usually considerably lowered by hemorrhage, yet the two deflections for a given flow checked with an error of not more than 3 per cent. This confirms observations on artificial systems that the calibration of the manometer is independent of the head of pressure over a rather wide range.

In order to measure flow into an organ by this method it is, of course necessary that all blood flowing past the constriction enter the organ being studied. For flow into the hind limb of the dog, we have placed the constriction on the aorta just above its bifurcation into common iliaes, cannulated one

iliac (after tying all intervening branches), and tied all branches on the contralateral iliac as far distally as the inguinal ligament (Fig. 2). Clotting in the cannula was less frequent if the stump of iliae to be cannulated was left long.

If it is assured that pressure just above the constriction rises and falls exactly with carotid pressure, the upper limb of the manometer may be connected with the carotid. In the work which we have done on blood flow through the leg as influenced by local effects, there is good ground for believing this to be true. In our heavily barbitalized or etherized dogs the difference in pressure in the carotid and the femoral artery, before constricting the aorta, was never greater than 1 mm. Hg, and remained constant through minor spontaneous changes in carotid pressure (mercury manometer connected with remaining carotid) such as occurred in the course of a half-hour's observation. Furthermore, even maximum changes in flow in one leg do not appear to affect the lateral pressure in the lowermost segment of the aorta under these conditions, since the differential was unaffected by completely occluding the remaining femoral artery. It was, therefore, concluded that, for our purposes, carotid pressure is a sufficiently accurate measure of pressure throughout the aorta down to the constriction. It is theoretically possible, however, that under some experimental conditions, such large deflections of flow may occur between the carotid and the lowermost segment of the aorta as to invalidate this technique. Where such deflections of flow may occur, the upper limb of the manometer should, for the sake of simplicity, be connected just above the constriction.

#### REFERENCES

- Merriman, M.: *Treatise on Hydraulics*, London, 1916, John Wiley & Sons.  
Daly, I. de B.: A Blood Velocity Recorder, *J. Physiol.* 61: 21, 1926.  
Wagoner, G. W., and Livingston, A. E.: Application of the Venturi Meter to Measurement of Blood Flow in Vessels, *J. Pharmacol. & Exper. Therap.* 32: 171, 1928.

# THE QUANTITATIVE DETERMINATION OF URINARY ESTROGENIC HORMONE (BIOLOGICAL METHOD)\*

A CONSIDERATION OF THE TECHNIQUE, ANIMAL COLONY, AND  
LABORATORY PERSONNEL

ALLAN PALMIL, M.D., SAN FRANCISCO, CALIF

INASMUCH as no chemical method of measuring estrogenic hormone has proved practical for the urine of nonpregnant women we must still rely upon a biological procedure for this purpose. Since the chemical identity of the estrogenic hormone in the urine of nonpregnant women has not, as far as I know, been accurately determined, it is safest to speak of such hormone as having estrogenic potency equivalent to a chemically pure hormone of known weight, preferably estrone. The value of determining the "free" and "combined" estrogenic hormone proportions in the urine of pregnant women and possibly also in the urine of nonpregnant women, has been demonstrated.<sup>1,2</sup>

The procedure to be outlined and discussed here has been developed from seven years of personal experience, with most of the described methods of biological assay, a knowledge of chemical methods, and from particular attention paid to the standardization of values obtained. Nearly a thousand urinary extracts have been prepared, of which 550 have been completely assayed. The procedure is described in two sections, the first dealing with the preparation of animals and the second with the preparation of urinary extracts. The method of preparing animals for testing is original and, except for a change in the grouping of animals, is identical with an earlier report.<sup>3</sup> No part of the technique on the preparation of extracts is original. It is a combination of the best features from several methods. The procedure can be applied to the urine of pregnant as well as nonpregnant women, men, and children.

## PROCEDURE

A *The Preparation of Animals*—On a Monday each of a hundred 4 month old female mice is castrated through a short dorsal incision, after which a skin clip is applied to allow healing of the wound. One week later, the skin clips are removed and each mouse is given a subcutaneous injection of 2 gamma estrone in 0.2 c.c. olive oil. On Monday of the next week each mouse is given 0.2 gamma estrone in 0.2 c.c. olive oil, and on the third day following a wire loop scraping of the vagina of each mouse is examined. Only those animals exhibiting complete vaginal cornification are set aside as eligible test animals.

\*From the Department of Obstetrics and Gynecology, University of California Medical School, San Francisco. Supported by the Christine Breon Fund for Medical Research.  
Received for publication June 6, 1938.

for unknown urinary extracts to be injected four days later, three weeks from the date of castration. The principle evolved here is one of overstimulating the animals. The dose given at the first injection (2 gamma) will cause every animal to go into estrus, consequently no examination of vaginal smears need be made. The dose of estrone given in the second injection (0.2 gamma) has been found to be the best minimally effective dose of estrone to which the most sensitive of animals will react. Early experiments revealed that while an animal would occasionally react to 0.1 gamma estrone, the percentages of animals responding to this small dose and the frequency with which the same animal would respond to the same small dose were so slight that it did not seem practicable to follow this earlier procedure. Admittedly then, the acceptance of 0.2 gamma estrone as the minimal effective dose introduced a possible error of 100 per cent at the outset, but this is minimized by the proper spacing of dose levels and by the use of three controlled animals for each test dose.

Animals not exhibiting estrus after the administration of the first 0.2 gamma estrone dose are overstimulated again with 2 gamma and given a second trial with 0.2 gamma estrone. To those animals still not responding to the minimal dose, the overstimulating procedure is repeated a third time before finally discarding nonresponsive animals. All injections, whether they be overdoses, minimal doses, or test doses of unknown extracts, are given routinely on Monday of each week. Vaginal smears of test animals only are examined Monday morning before injection, Wednesday morning and afternoon, Thursday morning and afternoon, and Friday morning. Full vaginal cornification in the animals under test, as revealed in the Thursday morning smear, is the only criterion of a positive reaction, and this is the same end point obtained when the known standard is used. The other smears from the animals under test are useful in checking, for evidence of persistent estrus and for error in castration.

After an animal has been used for testing an unknown extract, it is given the minimal dose of estrone a week later, provided it went into estrus with the unknown extract, or the overdose followed by the minimal dose if it did not go into estrus with the unknown. *Every animal used for testing must have responded in full estrus to the known standard minimal dose of estrone one week before.*

Three animal bins, each 1 foot by 2 feet by 1 foot, are maintained for each series of 100 castrated test mice. The first bin holds animals under test for the week, the second holds animals injected with 0.2 gamma estrone, and the third holds animals injected with 2 gamma estrone. Vaginal smears must be obtained from the animals in the first bin of each series on Monday, Wednesday, Thursday, and Friday; from the animals in the second bin of each series Thursday morning only; and not at all from the animals in the third bin of each series. Two or three bins are maintained for the newly castrated animals and as they respond for the first time to the minimal dose of estrone they are given numbers and used to fill in, as replacements, the missing numbers in any of the test animal series.

*B. The Preparation of Urinary Extracts.*—My method of estrogenic hormone extraction combines important features of several other methods.<sup>5-9</sup> Since it is suspected that sudden hormone excretion may occur at times during a period of a few hours or less, it is advisable always to require a complete twenty-four-hour urine specimen when an assay is to be done. This is particularly necessary when one wishes to demonstrate the daily excretion of hormone by any sort of a graph. I believe that a twenty-four-hour specimen of less than 800 c.c. with a specific gravity below 1.010 cannot be considered

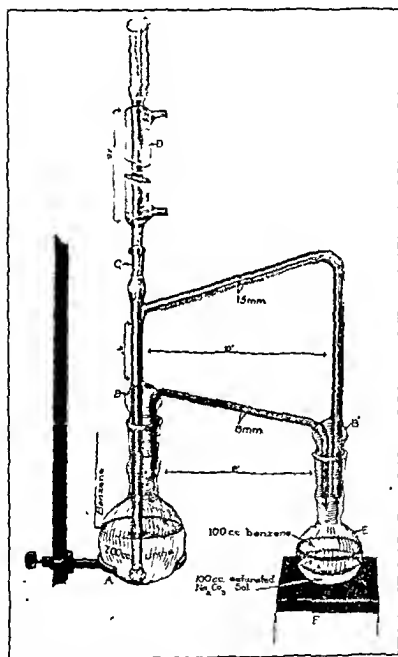


Fig. 1.—Continuous extraction apparatus (modified from Kurzrok and Ratner<sup>6</sup>). A, 1000 c.c. flask; B and B', standard groundglass coupling No. 35; C, standard groundglass coupling No. 15; D, water cooled reflux condenser; E, 500 c.c. flask; F, steam bath.

complete. The specimen should be collected in a jar containing about 2 c.c. of toluol, and if possible kept on ice during the period of collection. Toluol is a nontoxic preservative and does not interfere with quantitative gonadotropic hormone procedures. Any amount of estrogenic hormone that may go into the toluol preservative is automatically transferred to the "free estrin" extract. A maximum time limit, preferably twelve hours, should be adhered to between the time the collection of a specimen is completed and the time the



extraction of free estrin is started. This is of greatest importance with reference to the urine of pregnant women where slight bacterial hydrolysis is apt to occur in the urine on standing.

Each twenty-four-hour urine specimen is made up to 1,200 c.c. with distilled water if it does not already exceed that volume. Of this, 700 c.c. are used for estrin assay, and 500 c.c. set aside for gonadotropic hormone assay. The 700 c.c. portion is put directly into a continuous extraction apparatus (Fig. 1) and extracted with benzene for twenty-four hours. Chloroform, ether, ethyl acetate, and benzene have been tested for their efficiency in extracting internationally standardized estrone from aqueous solution. Of these solvents benzene has been found to be the most efficient. The collecting flask contains 100 c.c. of a saturated sodium carbonate solution as a wash for the benzene extract containing the hormone. Saturated sodium carbonate solution has been found not to remove estrogenic hormone from benzene solution. The latter is then transferred to a separatory funnel and washed with 100 c.c. of 2.5 per cent hydrochloric acid, saturated with sodium chloride, to remove any trace of alkali. A small amount of olive oil is added to the benzene solution and the benzene removed by steam heat. The final extract is made up to 7 c.c. with olive oil, thus making a 100-fold concentration of urinary estrin in its "free" state.

One hundred cubic centimeters of concentrated hydrochloric acid are next added to the 700 c.c. portion of urine and the mixture autoclaved for one and one-half hours under 15 pounds pressure at 120° C. After autoclaving, the urine is re-extracted with benzene for twenty-four hours, and the extract washed and evaporated as before and put into olive oil as a 100-fold concentration of urinary estrin in its "combined" state.

The olive oil extracts can be kept indefinitely without danger of deterioration. Assays have been carried out on the same extracts as long as two years apart, with identical values obtained.

Injections are made according to the form sheet in Table I and corrections for the twenty-four-hour volume calculated. Values for less than 4.0 gamma of estrone per liter of urine are not determined. When 4.0 gamma per liter cannot be demonstrated, the value is recorded as negative.

#### NORMAL VALUES FOR WOMEN

An absolutely accurate and full statement of normal values cannot yet be made due to the difficulties encountered in carefully collecting and assaying complete, consecutive twenty-four-hour urine specimens from truly normal individuals. However, it is safe to say that a normal woman excretes from 4 to 30 gamma of estrone-equivalent per day, except during the time she is menstruating, when the amount is usually not demonstrable. The excretion is greater during the second half of the intermenstruum. For one or two days at midintermenstruum there is normally a peak excretion that may amount to as much as 200 gamma. This peak is supposedly associated with ovulation. A consideration of the mechanism involved in ovulation and associated hormone excretion in a normal woman has been given previously.<sup>2</sup> A premenstrual rise in estrogenic hormone excretion is slight and inconstant.

TABLE I  
HUSARY 1 REACTION

HUSARY 1 COMMENTARY 24 HOUR VOLUME			HUSARY 1 EXTRACT DATA			
Extract (Whole)	Date injected	Dose	ANIMAL NO.	IN at HOURS	72 Hrs HOURS	RE HOURS
10/1	Date injected	Dose: 0.5 cc	1	1	1	
			2	1	1	
			1	1	1	Reaction
10/1	Date injected	Dose: 0.1 cc	1	1	1	
			2	1	1	
			1	1	1	Reaction
10/1	Date injected	Dose: 0.2 cc	1		1	
			2		1	
			1		1	Reaction
20/1	Date injected	Dose: 0.1 cc	1	1		
			2	1		
			1		1	Reaction
Extract (1:100 dilution)						
10/1	Date injected	Dose: 0.5 cc	1		1	
			2		1	
			1		1	Reaction
10/1	Date injected	Dose: 0.3 cc	1		1	
			2		1	
			1		1	Reaction
100/1	Date injected	Dose: 0.2 cc	1	1	1	
			2	1	1	
			1		1	Reaction
200/1	Date injected	Dose: 0.1 cc	1	1	1	
			2	1	1	
			1		1	Reaction
Extract (1:100 dilution)						
100/1	Date injected	Dose: 0.5 cc	1	1	1	
			2	1	1	
			1		1	Reaction
100/1	Date injected	Dose: 0.1 cc	1	1	1	
			2	1	1	
			1		1	Reaction
1000/1	Date injected	Dose: 0.2 cc	1	1	1	
			2	1	1	
			1		1	Reaction
2000/1	Date injected	Dose: 0.1 cc	1	1	1	
			2	1	1	
			1		1	Reaction
Extract (1:1000 dilution)						
1000/1	Date injected	Dose: 0.5 cc	1	1	1	
			2	1	1	
			1		1	Reaction
1000/1	Date injected	Dose: 0.1 cc	1	1	1	
			2	1	1	
			1		1	Reaction

gamma per 24 hours  
(calculated value - estimate equivalent)

Throughout the cycle of a normal woman the urinary estrin has been found to be in its combined fat-insoluble state. Only by extraction and assay of consecutive complete twenty-four-hour urine specimens from the same individual may the transient excretion of free estrin be demonstrated at mid-intermenstruum and again just premenstrually.

An excretion of less than 4 gamma estrone-equivalent per liter of urine seems to be definitely abnormal for a normal adult woman not bleeding. Since more than this amount has been found in some cases of persistent amenorrhea and natural menopause and since this amount has never been demonstrated in a patient whose ovaries have been removed, I believe that 4.0 gamma is an acceptable excretory level below which ovarian inactivity or absence of estrogenic hormone elaboration is quite definite.

#### LABORATORY HELP AND THE ANIMAL COLONY

Equipment and personnel for a laboratory to carry out routine hormone assays deserve much consideration. A statement as to the practicability of such a laboratory is not ventured. Two full-time technicians and a full-time animal caretaker are the minimum for the required number of persons devoting all of their time to this work.

An animal colony of not less than 1,000 mice should be maintained. Approximately 400 castrated adult female mice on hand at all times are necessary for the routine testing of twelve twenty-four-hour urine specimens each week. To maintain this number of animals for testing, it has been found necessary to castrate approximately 100 animals every three weeks.

The size of the animal colony advised is sufficient to allow almost an unlimited number of Aschheim-Zondek pregnancy tests and quantitative chorionic gonadotropic hormone assays.

A review of figures collected over a year's time reveals that the exposure of 40 adult female mice to males each week (1 male to 8 females) has been productive of an average of 17 litters of newborn mice per week. Too low a room temperature seems to have been the greatest single factor responsible for low fertility. This manner of breeding supplies an average weekly yield of 80 young females which can be used for pregnancy tests or quantitative gonadotropin testing when 21 days old. Those not used at 21 days maintain the strength of the colony for estrogenic hormone tests and for further breeding.

All but an extremely small percentage of the newborn males are destroyed when 2 or 3 days old. This allows a standard litter size of four to five young females to each mother. With the advent of a good rodent test for male sex hormone, the specifications for a laboratory described herein can be readily adjusted to supply animals for the quantitative determination of urinary androgen.

#### REFERENCES

1. Cohen, S. L., Marrian, G. F., and Watson, M.: Excretion of Estrin During Pregnancy, *Lancet* 1: 674, 1935.
2. Palmer, A.: Hormones in Urine of a Normal Non-Pregnant Woman, *Proc. Soc. Exper. Biol. & Med.* 37: 273, 1937.
3. Palmer, A.: Excretion of Hormone in a Case of Habitual Abortion, *Am. J. Obst. & Gynec.* 36: 1005, 1938.

- 4 Palmer, A. Method of Preparing Mice for Quantitative Determination of Urinary Estrogen, *Proc Soc Exper Biol & Med* 36 123, 1937
- 5 Smith, G. V., and Smith, O. W. Quantitative Determination of Urinary Estrin, *Am J Physiol* 112 340, 1935
- 6 Kurzrok, R., and Ratner, S. The Relation of Amenorrhea Accompanied by Genital Hypoplasia to the Follicular Hormone in the Urine, *Am J Obst & Gynec* 23 689, 1932
- 7 Schulowitz, M. J., and Wylie, H. B. Chemical Diagnosis of Pregnancy by Detection of Estrin in Urine, *J Lab & Clin Med* 21 210 1935
- 8 Cohen, S. L., and Marrion, G. F. The Hydrolysis of the Combined Forms of Oestrone and Oestrinol Present in Human Pregnancy Urine, *Biochem J* 29 1377 1935
- 9 Schulowitz, M. J., and Wylie, H. B. Micrographed supplement to reference No 7

## FULMINATE FERRICYANIDE REAGENT\*

WITH A NOTE ON A COLOR REACTION FOR THE FULMINATES

ROBERT D. BARNARD CHICAGO ILL

THE utility of direct titrations with ferricyanide solutions has been somewhat impaired by their pale color. Consequently their decolorization to ferrocyanide is an end point difficult to read. We have searched for an internal indicator to be used with the alkaline ferricyanide reagent for the time method of blood sugar determination of Hawkins and Van Slyke<sup>1</sup> as well as for the ferricyanide reagent employed in direct uric acid titration by the method of Barnard.<sup>2</sup> Only accidentally was it discovered that the fulminates (those of silver, sodium, and mercury were investigated in this connection) strike with ferricyanide, in neutral or alkaline solution a deep rose red color which appears characteristic for the fulminates. This coloration is evidently the result of the formation of an addition compound since the oxidant nature of the ferricyanide is not vitiated. The titer of the fulminate ferricyanide solution is equal to that of untreated ferricyanide solution as determined by titration of uric acid solutions.

The reagent is prepared by the addition of mercuric fulminate to potassium ferricyanide solution. It appears to keep indefinitely.

*Preparation of Mercuric Fulminate Suspension*—Ten grams of metallic mercury are dissolved in 10 cc of concentrated nitric acid and the resulting solution is poured into an equal volume of 90 per cent ethyl alcohol in a large Erlenmeyer flask. The reaction, which is rather turbulent is allowed to proceed under a hood. When complete the supernatant fluid is carefully decanted and the grayish deposit is washed with several changes of distilled water until all traces of acid are removed. The fulminate is kept under water. The danger in its handling has been considerably overestimated.

*Preparation of Centimolar Fulminate Ferricyanide Reagent*—0.329 gram of crystalline potassium ferricyanide is dissolved in about 50 cc of distilled water. A few crystals of the moist mercuric fulminate are added, and the

\*From the Laboratory of Therapeutics of the College of Medicine University of Illinois Chicago

reagent diluted to 100 c.c. The color intensity reaches its maximum in twenty-four hours, after which the reagent is ready for use. There should always be a slight excess of undissolved mercuric fulminate at the bottom of the container.

## REFERENCES

1. Hawkins, J. A., and Van Slyke, D. D.: Time Method for Determination of Reducing Sugars and Its Application to Analysis of Blood and Urine, *J. Biol. Chem.* 81: 459, 1929.
2. Barnard, R. D.: Electrometric Titration of Uric Acid, *J. LAB. & CLIN. MED.* 16: 1191, 1931.

1853 W. POLK STREET

## DEMONSTRATION OF THE BACTERIOSTATIC ACTION OF SULFANILAMIDE UPON HEMOLYTIC STREPTOCOCCI IN VITRO\*

ERWIN NETER, M.D., BUFFALO, N. Y.

FOLLOWING the important discovery of Domagk<sup>1</sup> of the therapeutic effectiveness of prontosil in experimental infections of mice with hemolytic streptococci, numerous articles have been published concerning various phases of this new form of chemotherapy of bacterial infections. It is beyond doubt that sulfanilamide and related compounds are effective also in the treatment of human infections due to hemolytic streptococci. In the first publication, Domagk emphasized that prontosil exerts its effectiveness only in vivo but not in vitro. In 1936, Colebrook and Kemy<sup>2</sup> reported that the serum of patients treated with prontosil retards the growth of hemolytic streptococci. In the same year, Colebrook, Buttle, and O'Meara<sup>3</sup> conclusively showed that sulfanilamide is bacteriostatic toward hemolytic streptococci; they found that small amounts of sulfanilamide (1:10,000) may delay the growth of hemolytic streptococci for several days, provided that a small number (30-50) of hemolytic streptococci were inoculated into the respective broths. When large numbers (30,000,000) of hemolytic streptococci were used, even a 1 per cent concentration of sulfanilamide failed to inhibit the growth of the organisms. Independently, Long and Bliss<sup>4, 5</sup> noted the bacteriostatic effect of sulfanilamide on hemolytic streptococci in vitro. Bliss and Long<sup>6</sup> furthermore demonstrated that a 1:1,000 diluted sulfanilamide lacked bactericidal effectiveness toward hemolytic streptococci suspended in broth or serum broth. The bacteriostatic effect of sulfanilamide in vitro toward small numbers of hemolytic streptococci was also corroborated by Osgood.<sup>7</sup> Mellon and Bambas<sup>8</sup> noted the marked bacteriostatic action of sulfanilamide upon hemolytic streptococci in spinal fluid of treated patients with streptococcal meningitis. It is important to state that the bacteriostatic action of a 1:1,000 diluted sulfanilamide is not demonstrable with all types of hemolytic streptococci. Bliss and Long<sup>9</sup> con-

\*From the Laboratories of the Children's Hospital and the Department of Pathology and Bacteriology, University of Buffalo School of Medicine.  
Received for publication, June 13, 1938.

clusively showed that such amounts of sulfanilamide failed to retard the growth of group D Lancefield hemolytic streptococci. This finding is of considerable clinical importance. In this connection it is of interest to mention that Helmholz<sup>10</sup> found the same of patients treated with prontosil, bactericidal toward staphylococci and various gram negative bacilli, but not toward enterococci.

It follows from this brief review of the literature that sulfanilamide is bacteriostatic toward certain strains of hemolytic streptococci. The demonstration of this bacteriostatic action however is a rather laborious procedure. The following is a description of a test tube method for the differentiation of sulfanilamide susceptible and sulfanilamide resistant strains of hemolytic streptococci. This method does not necessitate a dilution of the broth used for inoculation of the test medium nor a count of the number of microorganisms before and after treatment with sulfanilamide.

#### TECHNIQUE

Phenol red broth base (Difco) in the given amount was dissolved in neutralized distilled water, then 1 per cent soluble starch, 0.2 per cent agar, and 1 per cent maltose or dextrose, respectively, were added. The mixture was heated in a water bath until completely dissolved. Sulfanilamide powder was added to one part, the other part was used as a control. The sulfanilamide preparation used was prontosil, repurified for injection. After the sulfanilamide was dissolved by heating in a water bath, both broths were autoclaved for twenty minutes at 15 pounds pressure. The culture medium, referred to as phenol red broth and sulfanilamide phenol red broth, respectively, was always kept in the incubator at 37° C.

Various strains of hemolytic streptococci, isolated in this laboratory from human sources, were inoculated in phenol red broth containing sulfanilamide as well as in control broth. About one loopful of sixteen to eighteen hours' plain infusion broth culture was used for inoculation. The inoculated culture media were incubated at 37° C. Growth in the control phenol red broth, which is accompanied by change of the indicator from red to yellow due to the acid production, was used as the standard and compared with growth and acid production in the sulfanilamide phenol red broth. The difference in growth was taken as an indication of the bacteriostatic action of sulfanilamide upon the respective strain.

#### RESULTS

In the first experiment, 10 different strains of hemolytic streptococci were inoculated into (1) phenol red broth, containing 0.8 per cent sulfanilamide, and (2) phenol red broth control. Table I gives the results obtained. Of the 10 strains tested, 6 (group 1) were completely or markedly inhibited in sulfanilamide phenol red broth. Growth in the controls occurred after five to twelve hours' incubation at 37° C. After sixteen hours' incubation at 37° C, one of the 6 strains grew out in sulfanilamide phenol red broth, although the growth was markedly less than in the respective control. The remaining 5 strains failed to grow out in sulfanilamide phenol red broth even when incubated for two weeks. The 6 strains tested in this series were found to be

fibrinolytic and to produce in 1 per cent glucose broth a pH above 5.0. The remaining 4 strains (group 2) were not or only slightly inhibited in 0.8 per cent sulfanilamide phenol red broth. Three of these strains were identified as hemolytic enterococci by their growth on 40 per cent bile agar, their capacity to reduce methylene blue and litmus in skimmed milk, by their production of a pH below 4.8 in 1 per cent glucose broth, and by their ability to ferment mannitol. The remaining strain of this group, which was not inhibited by sulfanilamide, produced a pH of less than 5.0 in 1 per cent glucose broth and lacked fibrinolytic activity. This latter strain was isolated from the urine of a patient with cystitis.

TABLE I

BACTERIOSTATIC ACTION OF 0.8 PER CENT SULFANILAMIDE UPON HEMOLYTIC STREPTOCOCCI IN PHENOL RED BROTH

Strains number	GROUP 1						GROUP 2			
	674	1179	1276	1372	1373	2521	23	26	2640	2557
Phenol red broth with 0.8 per cent sulfanilamide	-	-	-	-	+	-	++++	++++	+++	+++
Phenol red broth as control	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

- = No growth or acid production.

+ to +++ = Various degrees of growth and acid production after sixteen hours' incubation at 37°C.

In order to test the action of various concentrations of sulfanilamide upon the growth of hemolytic streptococci, the following experiment was undertaken. Two strains of hemolytic streptococci (No. 1373 and No. 2521) were inoculated in phenol red broth containing various amounts of sulfanilamide: 0.8 per cent to 0.0000008 per cent (tubes a to g), respectively, and in phenol red broth without sulfanilamide as a control (tube h). The tubes were incubated at 37° C. The results are recorded in Table II. It may be seen from this table that both strains of hemolytic streptococci were inhibited in the presence of sulfanilamide. There were, however, quantitative differences to be observed. Strain No. 1373 failed to grow out in phenol red broth containing 0.8 per cent sulfanilamide; smaller amounts (0.08 per cent and less) were found ineffective, even after only eight hours' incubation at 37° C. On the other hand, strain No. 2521 was markedly inhibited in the presence of 0.000008 per cent sulfanilamide after eight hours' incubation; at this time, the

TABLE II

BACTERIOSTATIC ACTION OF VARIOUS AMOUNTS OF SULFANILAMIDE UPON HEMOLYTIC STREPTOCOCCI IN PHENOL RED BROTH

CONCENTRATION OF SULFANILAMIDE														
STRAIN NO.	0.8%		0.08%		0.008%		0.0008%		0.00008%		0.000008%		0	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1373	-	-	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
2521	-	-	-	±	-	++++	-	++++	-	++++	+	++++	+++	++++

- = No growth.

+ to +++ = Various degrees of growth and acid formation after: (a) eight hours at 37°C., (b) forty-eight hours at 37°C.

phenol red broth control showed profuse growth. After forty eight hours' incubation at 37° C, however, growth occurred in phenol red broth containing up to 0.008 per cent sulfanilamide. Substantially the same results were obtained in several experiments. It is important to emphasize in this connection that complete inhibition of growth of certain strains of hemolytic streptococci in the presence of 0.8 per cent sulfanilamide cannot account for the effectiveness of this drug in the treatment of streptococcal infections, since such concentrations are not obtained in treated patients.

In order to determine whether sulfanilamide delays or completely inhibits the growth of hemolytic streptococci, various strains were incubated in 0.8 per cent sulfanilamide phenol red broth for a period of three weeks. These experiments revealed that with some strains no growth occurred within this period of incubation, although the controls showed profuse growth after eight to eighteen hours.

# SUMMARY

1 The bacteriostatic action of sulfanilamide toward hemolytic streptococci is clearly demonstrable in a culture medium containing 1 per cent dextrose and maltose, respectively, 1 per cent soluble starch phenol red broth base, and sulfanilamide.

2 Employing this culture medium it was found that

- (a) Sulfanilamide is bacteriostatic toward various strains of fibrinolytic hemolytic streptococci, but not or only slightly, toward hemolytic enterococci isolated from man.
- (b) Sulfanilamide in a concentration of 0.8 per cent may continuously inhibit the growth of susceptible strains while smaller concentrations may retard their growth.
- (c) The result as to the sulfanilamide susceptibility of hemolytic streptococci in the described order of experiment may be obtained after eight to twenty four hours.

# REFERENCES

- 1 Domagk, G. Ein Beitrag zur Chemotherapie der bakteriellen Infektionen, *Deutsche med Wchnschr* 61 250, 1935.
- 2 Colebrook, L, and Kenny, M. Treatment of Human Puerperal Infections, and of Experimental Infections in Mice, With Prontosil. *Lancet* 1 1279 1936.
- 3 Colebrook, L, Buttle, G. A. H., and O'Meara, R. A. Q. Mode of Action of Para aminobenzenesulphonamide and Prontosil in Hemolytic Streptococcal Infections, *Lancet* 2 1323, 1936.
- 4 Long, P. H., and Bliss, E. A. Para amino benzene sulphonamide and Its Derivatives. *J. A. M. A.* 108 32, 1937.
- 5 Long, P. H., and Bliss, E. A. Observations Upon the Mode of Action and the Clinical Use of Sulfanilamide in Urinary Tract Infections. *South M. J.* 31 308, 1938.
- 6 Bliss, E. A., and Long, P. H. Observations on the Mode of Action of Sulfanilamide, *J. A. M. A.* 109 1524, 1937.
- 7 Osgood, E. E. Culture of Human Marrow. Studies on the Mode of Action of Sulfanilamide, *J. A. M. A.* 110 349, 1938.
- 8 Mellon, R. R., and Bumpus, L. L. Bacteriostatic Effect of Sulfanilamide in Spinal Fluid of Convalescent Cases of Streptococcal Meningitis, *Proc. Soc. Exper. Biol. & Med.* 36 682, 1937.
- 9 Bliss, E. A., and Long, P. H. The Failure of Para aminobenzenesulphonamide Therapy in Urinary Tract Infections Due to Group D (Lancefield) Beta Hemolytic Streptococci, *New England J. Med.* 217 18, 1937.
- 10 Helmholtz, H. F. The Use of Sulfanilamide as a Urinary Antiseptic, *J. Pediat.* 11 243, 1937.



## A SIMPLE INEXPENSIVE TURBIDIMETER\*

GERALD K. ASHBY, M.D., EVANSVILLE, IND.

SINCE a casual survey of the literature offered no description of a simple, inexpensive turbidimeter, it is thought that this note may be useful to other investigators. The apparatus described here is quite useful for rapid, approximate comparisons of turbidities, especially for bacteriologic work. Its value has been proved by use in standardization of bacterial spore suspensions prepared under like conditions and employed in disinfection studies.<sup>1</sup>

Smith<sup>2</sup> describes a turbidimetric method somewhat similar in principle to the one described here. However, the Nessler tubes used in his apparatus are unsuitable for extensive bacteriologic work because of their expense, compared to that of test tubes, and their inability to withstand heat sterilization. Other turbidimetric methods described use either the more elaborate Tyndall effect or photoelectric cells. These methods require more expensive equipment, but are better adapted for more accurate work.

Although only corrugated cardboard has been used by the writer, the turbidimeter may be constructed of wood or metal if a more durable apparatus is desired. It hardly requires mentioning that the use of corrugated cardboard enables one to make this turbidimeter in a short time without the aid of a mechanic or tools other than a ruler and a sharp knife.

Fig. 1 shows the corrugated cardboard body, *A*, in light outline; a test tube rack, *B*, and light source, *C*, in heavy outline. Behind the test tube, *D*, shown in place, is a screen, *E*, which is made from a full-sized sheet (8.5 by 11 inches) of medium weight typing paper, on which is ruled in ink a series of parallel lines of various weights or widths. The lines are best made with a ruling pen and India ink. However, a fountain pen and ordinary black ink will serve the purpose. The screen is securely fixed to the back of the test tube rack with gummed paper. To simplify construction, the test tube rack, also consisting of corrugated cardboard, is made separately from the body, slipped through a slot in the top of the body, and then permanently fastened in place with gummed paper.

A longitudinal slot, cut in the top of the body, that just fits the light socket adds to the accuracy of the apparatus, as it allows partial regulation of the light intensity so that it is possible to determine which of two tubes is first to extinguish a selected ruled line. If no provision is made for varying the intensity of the light source, the apparatus is limited to a range of turbidities through which may be seen the ruled lines on the screen. But, if bulbs of different

\*From the Bacteriological Laboratory, Mead Johnson and Co., Evansville.  
Received for publication, June 16, 1938.

wattages or a rheostat in series with a bulb are used, the apparatus becomes adaptable to a range of turbidities, varying from almost clear to hardly translucent.

The dimensions given in Fig. 1 may be varied to accommodate the ruled screen and the test tubes used.

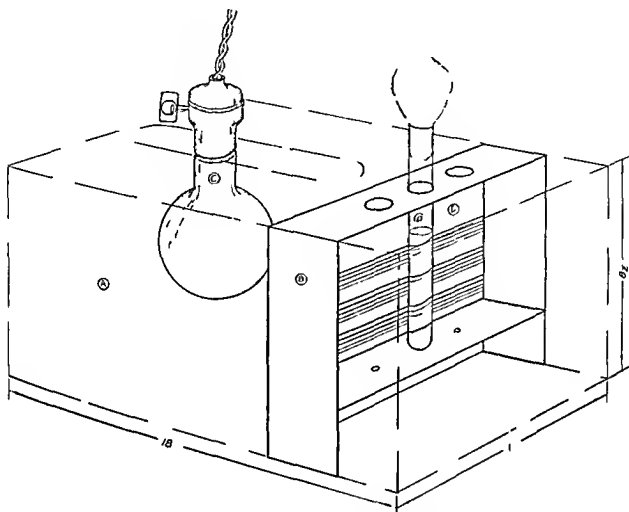


Fig. 1—A phantom perspective of the turbidimeter

Turbidity "standards" may be prepared from fuller's earth and distilled water, the proportions depending on the type of work to be done and the weight of lines used on the screen. The writer used one gram of fuller's earth to one liter of distilled water, allowed the heavier particles to settle for fifteen minutes and prepared subdivisions from the resulting suspension by mixing 2, 4, 6, 8, and 10 cc of suspension with 8, 6, 4, 2, and 0 cc of distilled water, respectively. The prepared standards are sealed with rubber stoppers and can be kept indefinitely, if one is careful to shake them well before each determination.

A more simple utilization of this same principle may be realized by merely suspending the ruled screen between the light source and the test tubes. But, under these conditions, the glare around the edges of the screen is an interfering factor and only fair results may be obtained.

#### REFERENCES

1. Ashby, Gerald K. A Study of the Effect and Efficiency of Germicides and Fumigants on Bacteria That Produce Ropiness in Bread. A thesis presented to the faculty of the Agricultural and Mechanical College of Texas in partial fulfillment of the requirements for the degree of Master of Science, Food Industries, July, 1938, p. 370. See also Ashby, G. K., Hedges, C. C., and Gibbons, E. H. *J. Bact.* 32: 84, 1937.
2. Smith, Walter E. Measurement of the Turbidity of Cane Juices, *Internat. Sugar J.* 27: 381, 1925.

# SULFOCYANATE AS A TREATMENT FOR MANGE ON DOGS AND OTHER LABORATORY ANIMALS\*

EMMETT B. CARMICHAEL, M.S., PH.D., UNIVERSITY, ALA.

FOR the past three years, we have been using a technical preparation of lauryl sulfoeyanate, "Loro,"† as a treatment for mange on laboratory animals. The "Loro" contains about 50 per cent of active ingredients and an emulsifying and wetting agent which allows one to use it in aqueous solution. Since the substance was so effective in curing the mange on guinea pigs,<sup>1</sup> we decided to experiment with it on dogs and other laboratory animals.

A 5 per cent aqueous solution is applied by means of a clean rag or sponge while the dog is standing in a large pan. It is better to wash the dog before the treatment, but it is not absolutely necessary. It is not essential to apply the solution to the whole surface of the animal if there are only a few small spots that have been affected by the itch mites. However, if the affected spots are numerous and generally scattered over the body, it is well to wet the whole animal with the solution. Since the dog can and does lick a great part of its body, it is well to hold the dog for about thirty minutes after the treatment, or use a muzzle which will prevent him from licking off the active substance. If the mange is of long standing and there are open sores or ulcers, it may take two or more treatments, but usually two are sufficient. We have treated dogs that had lost practically all of their hair and apparently had their whole body surfaces affected by the itch mites. In all such cases, we have been able to clear up the condition. If two or more treatments are to be used, they should be given about a week to ten days apart.

This treatment has been given to dogs that have lived in two localities: Birmingham and University. The dogs from the latter city were pets and were treated by their owners who have reported in every case that the bare, thickened, and inflamed areas were cleared up, and that hair began to grow within a short time.

A few cats and rats have been treated for mange with the "Loro" and they too have been cured. It is much easier to use on rats than to follow the treatment for scabies which was suggested by Greenman and Duhring<sup>2, 3</sup>; "In case of badly infested ears it is more expeditious and quite as effective to trim off the affected edges."

## REFERENCES

1. Carmichael, E. B.: Sulfoeyanate Treatment of Mange in Guinea Pigs, *Science* 63: 341, 1936.
2. Greenman, Milton J., and Duhring, F. Louise: Breeding and Care of the Albino Rat for Research Purposes, Philadelphia, 1923, The Wistar Institute of Anatomy and Biology, page 99.
3. Greenman, Milton J., and Duhring, F. Louise: Breeding and Care of the Albino Rat for Research Purposes, ed. 2, Philadelphia, 1931, The Wistar Institute of Anatomy and Biology, page 99.

\*From the School of Medicine, University of Alabama, University.

†E. I. du Pont de Nemours & Co., Wilmington, Del., supplied the "Loro."

Received for publication, June 17, 1938.

# A NEW INSTRUMENT FOR TESTING CONDITION OF MYOCARDIUM AT AUTOPSY (MYOCARDIOMETER)

DAVID B. FISHBACH, M.D., PHILADELPHIA, PA.

IN A recent article,<sup>1</sup> I briefly described early experiments with a new instrument for testing condition of myocardium at autopsy, with especial reference to degree of myocardial degeneration. This report is presented because the instrument has been modified so that the readings are more accurately and more easily ascertained and the various degrees of degeneration are presented with cases listed under chief cause of death.

Death due to myocarditis or true inflammation is comparatively infrequent, whereas myocardiosis or myocardial degeneration is found in most hearts of cases coming to autopsy. In deaths not due to cardiac causes the heart may be only slightly degenerated. In deaths due to heart condition, they may be explained on a physical basis of intrinsic mechanical defect, congenital or acquired, such as marked valvular stenosis, regurgitation, congenital anomalies, etc., or because of the poor propulsive power of the heart due to marked softening and degeneration of the myocardium itself. Heart failure may occur with or without lesions of the valves. There is (1) right sided, (2) left sided, (3) total failure. The classical example of right sided failure is mitral stenosis. Most of the cases reported here were either left ventricular failure or total failure.<sup>2</sup> Degenerations in the interventricular septum corresponded to degenerations of the left or right ventricular wall where there were no localized lesions due to coronary artery disease.

The myocardial fiber is a specialized cell, and is, therefore, subject to the usual degenerative changes from which cells suffer.<sup>3</sup> The arrangement of the musculature of the heart walls is such as to control with the greatest completeness the propulsion of the blood.<sup>4</sup> Degeneration of the myocardium occurs in many acute infectious diseases, such as diphtheria, scarlet fever, and others, it may be the result of poisons, such as phosphorus, arsenic, chloroform, ether, alcohol, and it may be due to anemia, primary or secondary. Grossly, the heart may be dilated and the muscle is likely to be soft, flabby, and pale. The cut surface bulges, and is more friable than normal. Practically never in infancy, rarely before the twentieth year, and increasing in frequency as age advances, the myocardium may show histologically numerous transverse fractures of the muscle fibers. It is usually stated that segmentation occurs in the line of the intercalated disks, and that a slightly different condition called fragmentation shows fractures of the fibers between and not necessarily involving the disks.<sup>5</sup> In segmentation the muscle fibers have separated at the cement line. In fragmentation the fracture has been across the fiber itself, and perhaps at the level of the nucleus.<sup>6</sup> Hearts the seat of marked fragmentation are lax, easily torn, the muscle fibers widely separated. In advanced cases of parenchymatous degeneration of the heart, it is very soft and friable. Softness means lessened

resistance to palpation, friability means easy separation and division of muscle fibers by pressure with the ends of thumb and finger. These two conditions usually, though not necessarily, accompany each other but are different attributes. A heart muscle may be soft in early degeneration but not friable, that is, it will not yield and allow the fingers to make holes in it by simple straight pressure.<sup>7</sup> Therefore, the more degenerated the heart muscle, the softer and more friable it will be.<sup>8</sup> Using an instrument that measures the amount of force required to perforate a portion of myocardium, the more degenerated the myocardium, the less the force required, and hence the lower the instrument reading will be. The less degenerated the myocardium, the more the force required to perforate it, and the higher the reading will be. Marked localized

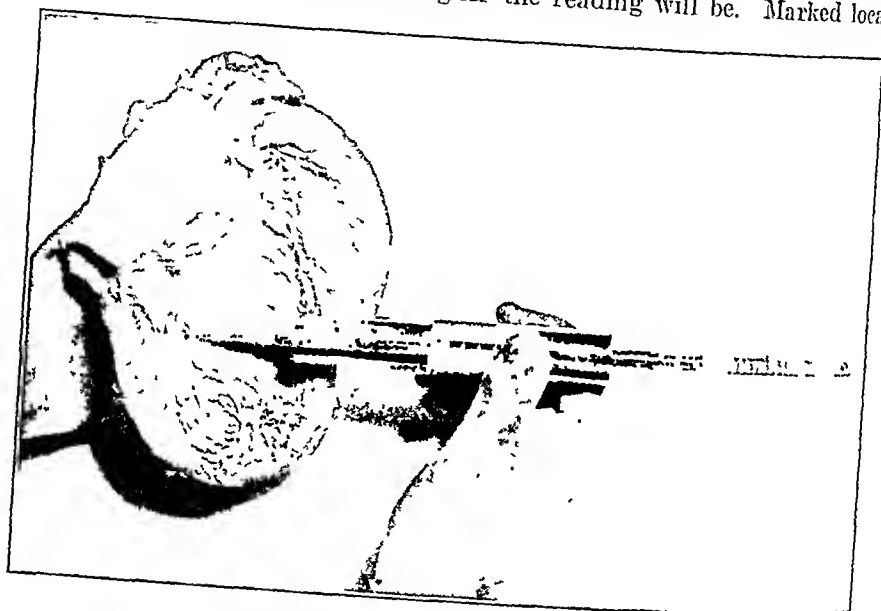


Fig. 1.—Performing test on heart muscle.

fibrosis would interfere with the accuracy of this test for myocardial degeneration. Large areas of localized fibrosis are usually due to coronary artery disease. Therefore, to perform the test in the heart, the center of the interventricular septum has been chosen because this area is least likely to show local changes due to coronary disease. This test is not designed to replace our previous methods of examining the heart at autopsy, but to add to our conception of the degree of myocardial degeneration. No longer is it sufficient to state that a heart shows myocardial degeneration; because a slight myocardial degeneration may but slightly have altered its function during life, whereas a marked myocardial degeneration must have altered the cardiac function to a greater extent. This instrument can be used at the time of autopsy in the gross examination of the heart to test degree of myocardial degeneration; so that one need not necessarily wait a week or more before histologic sections can be studied in order to determine the degree of degeneration. In most of the cases reported, the histologic examination of the cardiac muscle confirmed the gross findings of the myocardiometer.

Fatty degeneration arises from the same causes as, and is probably merely a later stage of, granular degeneration.<sup>9</sup> In cases of fatty degeneration of the heart, friability reaches an extreme degree, the wall being easily pinched through. In fatty infiltration of the heart, the affected muscle may be soft and friable but not to the degree of degeneration.

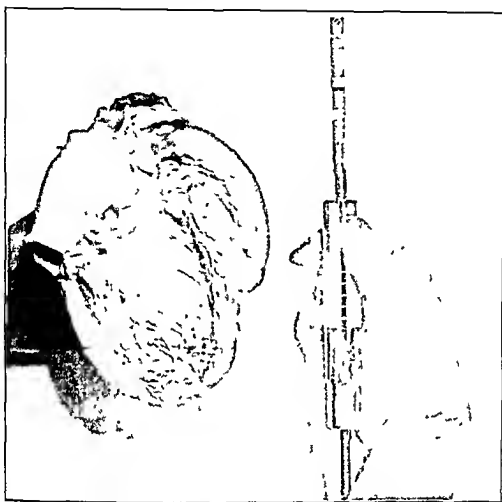


Fig 2—After test Note reading in grams pressure

The most marked degenerative changes in the heart muscle occur in necrosis<sup>10</sup> Necrosis of muscle fibers in the myocardium may occur from cutting off the blood supply completely by embolism or thrombosis followed by infarction, or partially as the result of narrowing of the lumina of arteries by thrombosis, or in consequence of arteriosclerosis. It may also follow the effect of diffusible toxins (toxic myocarditis) or result from the immediate presence of infectious agents (infectious myocarditis). In necrosis of the myocardium, there is a lateral swelling and rupture of the fibers histologically.<sup>10</sup>

The lowest readings with the instrument (meaning the most marked degenerative changes) occurred in those cases that showed necrosis of the heart muscle histologically. Post-mortem changes play but a negligible part in the heart muscle from the standpoint of consistency. Examination of a heart removed from the body four hours after death, and examination again eight hours after death, showed exactly the same myocardiometer reading of 700 gm. Twelve hours after death, in a case of hypertensive heart disease with heart failure, the temperature of the myocardium was 86° F., and the myocardiometer reading was 800 gm. Thirteen hours after death the temperature of the myocardium was 79° F., and the myocardiometer reading was 800 gm. Fourteen hours after death the temperature of the myocardium was 75° F., and the myocardiometer

reading slightly above 800 gm. Even a variation of 50 gm. is of very slight significance, since there is a wide range of readings to indicate moderate degeneration, marked degeneration, etc. Autopsies done thirty-six to forty-eight hours after death gave the usual myocardiometer readings, depending on the degree of degeneration.

The instrument is made of brass, heavily chrome plated and rust proof. There are two brass cylinders, one telescoping within the other. Inside is a brass spring, 1/16 inch gauge, tempered to a fixed hardness. At the distal end is a plunger, 6.25 by 0.5 mm. wide, over 3 cm. long, divided into centimeters. The space from 1 to 2 cm. is subdivided into millimeters, so that the thickness of the interventricular septum can be measured between these two readings. Inside of the brass cylinders is a brass graduated rod that projects from the proximal end of the instrument. The end of this rod rests against an indicator pointing to 0. During the test, when the plunger is inserted into the heart muscle (interventricular septum), the spring within the tubes becomes compressed, and the graduated rod is pushed outward pushing the indicator ahead of it. After the plunger has pierced completely the cardiac muscle, the spring recoils, the rod drops back, but the indicator remains in position, having a tiny spring on one side to keep it in position. The indicator shows the number of grams required to perforate that cardiac area. The readings are from 0 to 2000 gm., being subdivided into hundreds of grams, e.g., 0, 200, 300, 400, 500, etc.

#### TECHNIQUE OF TEST

1. The endocardium is scraped away from both sides of interventricular septum of heart, exposing the myocardium. If the septum is thicker than normal, the muscle is cut away until it is 12-15 mm. thick. A difference of several millimeters thickness of muscle alters the reading but slightly.

2. Holding the heart in the left hand and the instrument in the right hand, the plunger is pushed slowly through the center of interventricular septum.

3. When the plunger perforates the heart, the spring recoils, leaving indicator in position.

Reading is made in grams pressure required to perforate myocardium.

The more degenerated the heart muscle, the softer and more friable it is, and the lower the reading will be.

It is suggested that the degrees of degeneration be graded from grades 1 to 4.

	Gm.	
Normal hearts (accident cases, etc.)	1800	Grade 1
Slight degeneration	1300-1800	Grade 2
Moderate degeneration	900-1300	Grade 3
Marked degeneration	600- 900	Grade 4
Very marked degeneration	Below 600	

Readings of 1300 are considered as moderate degeneration.

Readings of 900 are considered as marked degeneration.

When the reading is 900 or below, congestive heart failure has usually taken place, and in the absence of marked changes in the other vital organs, it is considered to be a cardiac death due to advanced myocardial degeneration.

A total of 145 cases is presented, 78 males and 67 females

Grade 3 and grade 4 myocardial degeneration is severe enough to have caused death, in the light of our present knowledge Grade 2 myocardial degeneration may have been a cardiac death, if there were marked local degenerative changes due to coronary artery disease, or if there were intrinsic mechanical defects, such as marked stenosis, congenital anomalies, etc

Of the 145 cases, the hearts showed the following degrees of degeneration 5 normal, 31 slight degeneration, 58 moderate degeneration 38 marked degeneration and 13 very marked degeneration

Myocardiometer Readings (grains pressure)

Normal Hearts												
Skull fracture	1800	1800	1800	Spleen rupture				1900	Brain tumor			1800
Hypertensive Heart Disease												
Benign hypertension			Malignant hypertension			Chronic glomerular nephritis						
1300	1300	1000	1250	800	1450	700	1100	800	700	1000		
Acute Rheumatic Heart Disease												
				900	1150	1200						
Coronary Thrombosis												
	700	900	1100	900	1100	1250	1200	800	800			
Subacute Bacterial Endocarditis												
				1200	700	1250						
Myocardosis (due to toxic factors in chest abdomen brain etc.)												
1600	800	800	600	1300	1500	1700	1000	1500	1600	1450	1500	
1000	500	600	500	1300	900	600	600	900	1300	1450	900	
1100	750	800	900	1600	1300	1150	700	900	1100	1200	1250	
1000	600	800	600	1300	800	Gris bacillus septicaemia				400	450	
Carcinoma												
Breast	1600	1300	900	Head of pancreas				1100	1250	1100		
Uterus	1500	1100	1200	Lung				1500	900			
Prostate	1300	1600	1100	600	800	Liver (primary)		900				
Urinary bladder	1300			Stomach		700	900	1500	1450	1100	1300	
Testicle	1300			Large intestines				1500	1500	1050	1450	
Sarcoma												
	Bone	1200	1500	Lymphosarcoma				1200				
Lobar Pneumonia												
1100	1300	1100	1100	1500	1800	1100	1300	1150	1500	1500	1000	
Bronchopneumonia												
700	600	700	900	900	1100	1100	1200	1300	900	900	1100	
				1800	1400	1700	1500					1200
Pulmonary Tuberculosis												
Acute Myelogenous Leucemia												
Lymphocytic Angina												
				550					600			

To summarize it, hypertensive heart disease showed grade 1, 3 cases, grade 2, 4 cases, grade 3, 4 cases Acute rheumatic heart disease showed grade 2, 2 cases, grade 3, 1 case Coronary thrombosis showed grade 2, 4 cases, grade 3, 5 cases Subacute bacterial endocarditis showed grade 1, 1 case, grade 2 1 case, grade 3, 1 case Carcinoma showed grade 1, 10 cases, grade 2, 16 cases, grade 3, 6 cases, grade 4, 1 case, this last case showing extensive suppuration within the stomach malignancy Lobar pneumonia showed grade 1, 4 cases, grade 2 8



cases. It is interesting to note here that not a single case of the 12 lobar pneumonias showed marked or very marked myocardial degeneration. Bronchopneumonia presented a different picture, grade 2, 10 cases; grade 3, 8 cases; grade 4, 1 case. Pulmonary tuberculosis showed grade 1, 4 cases.

#### SUMMARY

A new instrument has been devised to test condition of heart grossly at autopsy, thus aiding in a more accurate way our conception of the degree of degeneration of the heart muscle.

Histologic examination of sections of heart from the site of test corroborated the gross findings as to degree of myocardial degeneration.

One hundred and forty-five cases were studied at autopsy, 78 males and 67 females.

A much greater series of cases must be studied in order to ascertain the degree of degeneration in various types of diseases.

It is suggested that slight myocardial degeneration be called grade 1 degeneration; moderate, grade 2; marked, grade 3; very marked degeneration, grade 4. Grades 3 and 4 myocardial degeneration can be assumed to be cases of primary cardiac death in the absence of marked pathology in the other vital organs (lung, brain, etc.).

Average heart readings showed less myocardial degeneration in lobar pneumonia than in bronchopneumonia. This applied to the cases of bronchopneumonia which preceded cardiac failure and were not terminal bronchopneumonias on a pre-existing pulmonary congestion and edema.

Average heart readings showed slight or very slight degeneration in pulmonary tuberculosis.

The lowest myocardiometer readings, with the most marked degree of generalized myocardial degeneration with necrosis of heart muscle, were found in two cases of gas bacillus septicemia (*B. aerogenes capsulatus* or *B. welchii* infection).

#### REFERENCES

1. Fishback, D.: A New Instrument for Testing Cardiac Condition at Autopsy, *J. Lab. & Clin. Med.* 22: 952, 1937.
2. Warfield, Louis M.: The Treatment of the Patient Who Has Heart Failure, *Wisconsin M. J.* 34: 546, 1935.
3. Boyd, W.: Text Book of Pathology, ed. 2, Philadelphia, Lea & Febiger, p. 411.
4. MacCallum, W.: Text Book of Pathology, Philadelphia, W. B. Saunders Co., p. 448.
5. Karsner, H. T.: Human Pathology, Philadelphia, J. B. Lippincott Co., pp. 437-439.
6. Osler, W., and McCrae, T.: Principles and Practice of Medicine, New York, D. Appleton & Co., p. 807.
7. Ellis, A. G.: Elements of Pathology, Philadelphia, P. Blakiston's Son & Co., pp. 321-322.
8. Delafield, F., and Prudden, T. M.: Text Book of Pathology, ed. 15, New York, William Wood & Co., p. 621.
9. Coplin, W. M. L.: Manual of Pathology, ed. 5, Philadelphia, P. Blakiston's Son & Co., p. 490.
10. Mallory, F. B.: Principles of Pathologic Histology, Philadelphia, W. B. Saunders Co., pp. 424-425.

2806 FRANKFORD AVENUE

The cases included in this report were from the Jewish Hospital, Philadelphia, and Temple University Pathology Department.

I wish to thank Dr. L. W. Smith, Dr. J. C. Doane, Dr. E. S. Gault, Dr. F. Konzelmann, Dr. H. C. Lennon, and Dr. E. E. Aegerter, for their helpful suggestions and some of the figures

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**MONONUCLEOSIS, Infectious, False Positive Wassermann Reaction in, Bernstein, A** *Am J M Sc* 196 79, 1938

Transitorily positive serologic tests for syphilis were encountered in 6 out of 37 cases of infectious mononucleosis. During the acute stage of the disease the Wassermann test, the Eagle test, or both reactions, became positive to high titers, but spontaneously reverted rapidly to negative. These positive tests are independent of the presence of sheep cell antibodies in the patient's serum and indicate, as does the occasional occurrence of miscellaneous bacterial antibodies, the versatility of antibody responses in infectious mononucleosis. In any instance of apparent purpura hemorrhagica with a false positive Wassermann reaction, a Paul Bunnell test should be performed in order to establish the possible diagnosis of the thrombocytopenic variety of infectious mononucleosis. Reasons are stated for suspecting that the causative agent of infectious mononucleosis may be a protozoan.

**BLOOD New and Simple Method for Detection of, by Heating, Moody, A. M., Proescher, F., and Carr, J. L.** *Arch Path* 26 501, 1938

Study of the physical changes which occur when blood and a few other organic substances are heated to high temperatures has demonstrated that dried blood melts at from 500° to 515° F (260° to 268° C), and that blood after further heating above 700° F assumes a specific structure. This structure is easily identified in quantities as small as 1 mg, even when the blood has been mixed with inorganic or other organic substances. The physical changes are specific, not only for blood, but also for the degree of heat used.

**SPUTUM Quantitative Method for Estimating the Number of Tubercle Bacilli in, Jordan, E. F.** *Am Rev Tuberc* 38 241, 1938

The sediment from the centrifuge tube in which the mixture of sputum and 4 per cent sodium hydroxide had been spun for thirty minutes at high speed is washed with sodium chloride solution into a small test tube, which has a volume of 15 cc indicated by a file mark. The centrifuge tube is twice rinsed with a small amount of salt solution, and the rinsings are added to the sample. The volume of the combined sediment and rinsings is then made up to 15 cc by the addition of saline solution. Three or four glass beads are added to the tube which is then stoppered and agitated in the shaking machine for thirty minutes. (Others in the laboratory have found that a hard shaking by hand for five minutes is equally satisfactory.) The slides are marked, as Hughes suggests, with a diamond pencil line drawn 15 mm from one end across a slide 1 inch wide. After cleaning with acid alcohol, a loopful of sterile serum is spread over the area marked off. The serum dries almost immediately and serves as a fixative. A measured amount of the digested concentrate is added, using a pipette graduated in hundredths of a cubic centimeter. This is spread as evenly as possible over the layer of serum. For the first count, four slides are made from each specimen with 0.02, 0.04, 0.06, and 0.1 cc, respectively. This is done in order to obtain a slide in which there are 10 to 25 bacilli in each field. Fixing and staining are carried out in the usual manner.

In examining the slides under the microscope, a preliminary inspection is made to ensure that there is a fairly even distribution and that the slide is free from clumps of bacilli which in some specimens are especially troublesome. Elastic tissue may enmesh an enormous number

of microorganisms and prevent an accurate count, and occasionally bacilli are grouped as if agglutinated. Of the 38 concentrates examined, 3 were found to be uncountable for one of the above reasons.

If the slides were not obviously uneven, two groups each of ten fields were counted. The computation of these results is very simple. The mean of ten fields is determined. If the slide was made with 0.02 c.c., the mean is multiplied by 75; if 0.04 c.c. was used, 37.5 is the factor; if 0.06 c.c. was used, 25 is the factor; and for 0.1 c.c., 15, which gives the number of microorganisms per microscopical field in 1.5 c.c., is the volume of the concentrate. The number of microscopical fields in the area on the slides is determined, as in the Hughes method, by establishing the diameter of the field in use with a hemocytometer and solving the formula  $\frac{1}{4} \pi D^2$  for the area of the microscopical field. The area of the slide which the diluted concentrate is spread over is 25.4 mm. by 15 mm., or 381 sq. mm.  $\frac{1}{4} \frac{381}{\pi D^2} = 31046$ , the number of microscopical fields per slide. This must of course be determined for each microscope. For simplicity we have used 31,000, so our formula is:

$$\text{Mean} \times \frac{1.5}{\text{Amount of material used on slide in c.c.}} \times 31,000 = \text{Number of bacilli in sputum.}$$

Since the concentrate contained the tubercle bacilli from a seventy-two-hour sample, the result of the formula is in terms of the excretion for that period. It was not converted into twenty-four-hour excretion because we wished to use the Gaffky on these specimens for comparison.

**ENDOCARDITIS**, Experimental Streptococcic, Kinsella, R. A., and Muether, R. O. Arch. Path. 62: 247, 1938.

Seventeen dogs were subjected to operation whereby the mitral valve or the chordae tendinae were cut. All these animals were then fed with living cultures of nonhemolytic streptococci either mixed with food or by stomach tube. Ten of the animals became sick, displayed positive results of blood culture, and died. At autopsy these infected animals had bacterial endocarditis. The bacteria in the vegetations were determined to be identical with those that had been fed to the animals.

Streptococcic endocarditis can be produced in dogs with injured cardiac valves by feeding them streptococci.

The fact is thus established that bacteria entering the animal body through the mouth may become implanted on an injured area within the body. The exact route which these bacteria follow is not determined.

The reproduction of streptococcic endocarditis is complete. The success of two different drugs in curing the disease in dogs, while failing to cure the disease in human beings, does not obscure the identity of the experimental disease. On the other hand, the difference in the mode of production of the experimental disease and in the mode of production of the disease in human beings is emphasized. This difference lies in the fact that bacterial implantation begins after an injury produced by trauma in the one instance and as a result of disease in the other. It seems highly important to collect a series of animals cured of streptococcic endocarditis and then to study the treatment after reinfection of the healed scars of previous infection. This will be a tedious task. The most interesting by-product of the present study has been the demonstration of infection of traumatized valves by means of feeding streptococci to the animal. This part of the work, repeated in different years, seems adequately authenticated. It is of further interest that none of the microscopic appearances in the heart or elsewhere was such as to suggest a relation between the lesions observed in dogs and those of rheumatic fever which appear in human tissues.

**ANEMIA of Alcohol Addicts**, Bianco, A., and Jolliffe, N. Am. J. M. Sc. 196: 414, 1938.

A quantitative study of the red blood cells in 184 cases of alcohol addiction is reported. Of these subjects, 159 were "complicated" in that they had, in addition to alcohol addiction, one or more of the following diseases: polyneuritis, pellagra, "alcoholic" stom-

titis, "alcoholic" encephalopathy, or liver cirrhosis, 25 of these subjects were "uncomplicated" in that they had none of the above listed diseases or other recognized stigmas of chronic alcoholism

In these subjects, quantitative anemia did not occur in the "uncomplicated" group, but was present in 61 per cent of the "complicated" group. However, macrocytosis was present in about 50 per cent of both groups. Macrocytic anemia in these alcohol addicts was not limited to subjects manifesting pellagra or cirrhosis of the liver, but occurred as well and in about the same frequency, in subjects having polynecrosis, "alcoholic" stomatitis and "alcoholic" encephalopathy. There was also no correlation between the frequency of macrocytosis and achlorhydria, severity of liver damage, or the presence of an enlarged liver. In view of these findings the authors are inclined to regard the macrocytosis of the alcohol addict not as a manifestation of inability on the part of the liver to store a hemopoietic principle but as an extrinsic deficiency of some necessary hemopoietic substance required to maintain normocytosis.

**E COLI, Ingestion of Red Blood Cells by, and Its Significance in, Tyzzer, E. E. and Gelman, Q. M. Am J Hyg 28 271, 1938**

The present study was initiated by a problem in diagnosis arising from a case of brief intestinal obstruction which presented a discharge of blood-streaked mucus in which were numerous endamoebae with ingested red blood cells.

A thorough study of the amoebae present, taking into account morphology of trophozoite and cyst, growth on culture media, and the results of animal inoculation, failed to furnish any evidence of the presence of *E. histolytica*, but showed conclusively that red blood cells were being ingested by *E. coli*. On consideration of pathologic conditions other than amoebiasis to account for the continued presence of blood in the stool, a papillomatous polyp of the sigmoid was located and removed.

The observations of Dobell in regard to the ability of *E. coli* to ingest red blood corpuscles *in vitro*, find application in the occurrence, under certain circumstances, of red blood cells in this species as it is passed from the human intestine.

The acceptance of the presence of amoebae with ingested red blood cells as sufficient, without further morphologic study, for a diagnosis of amoebic dysentery may be expected to lead to erroneous diagnosis involving not only failure to recognize conditions actually present but also the subjection of patients to costly and unnecessary treatment.

**WHOOPIING COUGH, Diagnosis of, Donald, A. B. Brit M J Sept 17, p 613, 1938**

An attempt has been made to assess the value of the various methods available for the early diagnosis of whooping cough. The methods are: 1. Specific tests—(a) cough plate method, (b) complement fixation tests, (c) intradermal tests, 2. Non specific tests—(d) total and differential leucocyte count, 3. Erythrocyte sedimentation rate.

**Specific Tests**—(a) The cough plate method is the only means of certain diagnosis in the earliest stages of the disease. In a series of 136 cases at all stages, 100 per cent were found to give positive plates in the first week, 93.5 per cent in the second week, 94.8 per cent in the third week, and 44 per cent in the fourth week, after which there is a rapid fall to 7.1 per cent in the fifth week, and less than 1 per cent in subsequent weeks. The value of the cough plate in the diagnosis of atypical and abortive cases is stressed, and its value to public health administrators is mentioned. Emphasis is also laid on the negative cough plate as a criterion of the termination of infectivity.

(b) The complement fixation test is shown to be of little value except as a means of corroboration of the diagnosis, and occasionally as an indication of immunity. In a series of 123 cases the reaction was found to become positive in 25 per cent during the third week of illness, and reached a maximum during the eighth week, when 89 per cent of the cases gave positive fixation reactions.

(c) The intradermal test, using a suspension of *H. pertussis*, is found to be valueless in diagnosis or as a method of determining susceptibility or immunity.

## REVIEWS

---

Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

---

### Medical Applications of the Short Wave Current\*

IN THIS book Bierman and Schwarzschild present a clear, sane, and logical exposition of the fundamental aspects of short wave therapy and an excellent and comprehensive discussion of its clinical applications.

---

### Manual of Veterinary Bacteriology†

THE third edition of this excellent manual deserves as cordial a reception as its predecessors and takes their place as the standard reference text in this field. Lieut. Col. Kelsor's book can be heartily recommended.

---

### The Synovial Membrane and the Synovial Fluid‡

IN THIS book, the author, formerly research fellow in the Hospital for Joint Diseases, New York, presents the digested results of fifteen years' research on the structure of the synovial membrane, the origin and nature of the synovial fluid, and the composition of pathologic joint effusions.

Part I (Chapters I-VII) is devoted to a discussion of the synovial membrane. Part II (Chapters VIII-XIV) discusses the synovial fluid; and Part III (Chapters XV-XXIV) discusses clinical applications.

---

\*Medical Applications of the Short Wave Current. By William Bierman, M.D., Attending Physical Therapist, Mt. Sinai Hospital, New York City. Including a Discussion of the Physical and Technical Aspects. By Myron M. Schwarzschild, M.A., Physicist, Beth Israel Hospital, New York City. Cloth, 379 pages, 25 plates, \$5.00. William Wood & Co., Baltimore, Md.

†Manual of Veterinary Bacteriology. By Raymond A. Kelsor, D.V.M., A.M., Ph.D., Lieut. Col. Vet. Corps U. S. Army, Chief, Veterinary Division, Surgeon General's Office, War Department, Washington, D. C. Cloth, 640 pages, 93 illustrations, \$6.00. Williams & Wilkins Co., Baltimore, Md.

‡The Synovial Membrane and the Synovial Fluid, With Special Reference to Arthritis and Injuries of the Joints. By David H. Kling, M.D., Assistant Professor of Orthopedic Surgery and Chief of Arthritis Clinic, White Memorial Hospital, Los Angeles, Calif. Cloth, 299 pages, 80 illustrations, \$5.00. The Medical Press, Los Angeles, Calif.

# The Journal of Laboratory and Clinical Medicine

VOL. 24

APRIL 1939

No 7

## CLINICAL AND EXPERIMENTAL

### THE BLOOD GLUTATHIONE IN HEMATOLOGIC DISEASES\*

E V KANDEL, M.D.,† AND G V LEROY, M.D.  
CHICAGO, ILLINOIS

THE discovery that cysteine containing proteins are necessary for growth was the basis for some questionable reasoning concerning glutathione. It was assumed by some workers that since cysteine differed from other amino acids chiefly in the possession of a sulphhydryl group ( $-SH$ ), then any compound having this group should display similar properties. In 1921 Sn F Gowland Hopkins demonstrated that reduced glutathione was responsible for the active reducing properties that some tissues possessed. There has been, since then, not only a great amount of speculation about the role of glutathione, but also there has been sound scientific work. Glutathione, glutamyl cysteinyl aminoacetic acid, is present in all the tissues of the body. Those tissues that are the most cellular contain the greatest amounts. Very little of the substance is present in blood plasma or in the extracellular fluids. The compound exists in the body chiefly as reduced glutathione (GSH). It readily parts with its hydrogen atom (from the sulphhydryl radical) and two molecules of GSH join to form "quasi oxidized" or disulfide glutathione (GSSG). Further oxidation breaks up this compound, and the sulphhydryl becomes successively  $-SO$ ,  $-SO_2$ ,  $-SO_3$ , and  $-SO_4$ . The oxidation reduction system  $2GSH = GSSG + 2H$ , is the reaction of the compound of greatest interest to biological chemists.

In a recent study of biological oxidation Laman and Barron<sup>1</sup> stated

"It is surprising to find that, although the role of glutathione as a respiratory catalyst was put forward as soon as Hopkins discovered it 16 years ago, there is as yet no positive evidence that glutathione functions as such."

\*From the Hematology Clinic Department of Medicine University of Chicago

†Douglas Smith Foundation Fellow

Received for publication August 13 1938

They further remarked that:

"Glutathione and ascorbic acid seem to possess closely related properties in the chemical activities of biological systems, maintaining graded levels of reduction intensity, necessary for the performance of certain biochemical processes. Both substances are present in the living cell mostly in their reduced form, both are catalytically oxidized by copper, and glutathione, like ascorbic acid, is oxidized by atmospheric oxygen with hemin and hemochromogens as catalysts."

In this connection, Hopkins and Morgan<sup>2</sup> have demonstrated that glutathione can prevent the oxidation of reduced ascorbic acid by the hexoxidase of Szent-Györgyi until all the glutathione present is oxidized. Furthermore, oxidized ascorbic acid is slowly reduced by reduced glutathione. In the presence of this hexoxidase, the reduction occurs five times as rapidly. Similarly, reduced glutathione protects ascorbic acid from oxidation by copper catalysis.

Two other observations on the chemical action of glutathione may be mentioned, although they have not been interpreted yet. Tait and King<sup>3</sup> noted that reduced glutathione increased the rate of oxidation of lecithin and other fatty substances. Meldrum and Tarr<sup>4</sup> demonstrated that oxidized glutathione (GSSG) is reduced anaerobically by the Warburg-Christian enzyme-coenzyme system in the presence of hexosemonophosphoric acid.

Contrary to the opinion of Lyman and Barron, Handovsky<sup>5</sup> presented evidence to indicate that glutathione acts as an emergency auxiliary respiratory ferment whenever the oxygen supply to a cell becomes either relatively or absolutely inadequate. His reason for so thinking was that he observed increased amounts of GSH in tissues poisoned with hydrocyanic acid, or exposed to a decreased oxygen tension. This idea, incidentally, tended to support the work of Gabbé<sup>6</sup> who found that in experimental anemia the glutathione quotient  $\frac{(\text{GSH in mg. per cent})}{(\text{R.B.C. in millions})}$  increased. This occurred when the animals

were bled rapidly, or poisoned with phenylhydrazine; and also when they were kept in an environment with a reduced oxygen content.

Of more interest in relation to the hypothesis to be described presently, are two directly contradictory studies. Bumm and Appel<sup>7</sup> reported that reduced glutathione reversibly and specifically inhibited the Pasteur reaction in tissue. (The Pasteur reaction is the inhibition of aerobic glycolysis by respiration, i.e., in the presence of adequate oxygen the conversion of glucose to lactic acid does not occur.) Their data showed that glutathione had no effect on respiration or on anaerobic glycolysis of tumor tissue; but that it increased the aerobic glycolysis to the anaerobic level. If such a conclusion could be verified, it would suggest that glutathione might serve to regulate the Pasteur-Meyerhof cycle in muscle. Then if increased amounts of glutathione could be demonstrated in tumor tissue, this action might explain the break in the Pasteur reaction which occurs in tumors. However, Baker<sup>8</sup> repeated the study and reported that reduced glutathione had no significant

effect on the aerobic glycolysis of tumor, brain, testis, and embryo tissue. She concluded that GSH neither participates in nor affects the Pasteur reaction.

The faulty syllogism which yielded the concept that the  $-SH$  radical influenced growth, gave rise to another equally questionable idea. This was, that since cancer is an overabundant growth of tissue, it may be due to a local accumulation of glutathione. Although there is considerable variation in the analyses of neoplasms for glutathione the majority of workers have not found a significant excess. As a matter of fact, even if such a result were always found, the remark of Hinep<sup>9</sup> in his excellent survey of the significance of sulphhydryl as a growth factor would be pertinent. He pointed out that

"The mere fact that a certain substance is accumulated in proliferating tissues is, moreover, insufficient proof of its relation to cell multiplication."

The exponents in America of the theory that growth—in particular malignant growth—is somehow related to the sulphhydryl radical have been Hammett and Reimann. In general this theory argues that sulphhydryl as the substance occurring naturally in the cell, is the universal and essential stimulus to growth. The reaction product derivatives of  $-SH$  are the natural inhibitors of this process, so that the stimulation by  $-SH$  is normally limited by the inhibitory action of the suboxidized sulfur forms of  $-SH$ . These are  $-SO$ ,  $-SO_2$ ,  $-SO_3$ , resulting from the normal oxidation of sulphhydryl. Cell proliferation is, therefore, regulated by the intracellular equilibrium  $-SH$   $-SO_3$ . Other mitosis stimulating agents are said to work through sulphhydryl. Experiments of others that failed to demonstrate a disturbance of this equilibrium, and recent genetic studies have led to a slight change in Hammett's<sup>10</sup> views, namely, that the potentiality for malignancy lies in an hereditary determination of lines of cells having (as in the embryo) a heightened sensitivity to sulphhydryl. Recently, experiments with carcinogenic hydrocarbons and para thioresol (which contains the  $-SH$  group) have suggested to Reimann and Hall<sup>11</sup> that sulphhydryl may exert a protective action against carcinogenesis. How this dilemma will be resolved is not yet clear.

It was inevitable that such a theory of canceration would be examined clinically. Nothing will be gained by reviewing all of the papers bearing on this problem, but two studies should be mentioned. Both of these were performed on blood because it is readily available and in the hope that changes in it would accurately reflect variations in the composition of tissues with respect to glutathione. Many analyses for glutathione have been made in the blood of patients with cancers. In general the content has been low and this has been attributed to an absorption or a fixation of glutathione by the malignant tissues. Schoonover<sup>12</sup> examined the blood of untreated and treated cancer patients and also normal subjects. The average reduced glutathione value for the diseased group was not significantly different than the normal controls. (This coincides with our experience in the case of blood dyscrasias.) She then estimated the ratio GSSG/GSH in the blood plasma in the erythrocytes and in the whole blood. In the patients with cancer, treated or not,



this ratio deviated from the normal. How significant this deviation was, is not stated. The plasma and the erythrocyte GSSG:GSH ratios were then divided into each other, giving even larger (numerically) discrepancies between the normal and the cancerous subjects. The significance of these observations is not clear, but it is worth recalling that Gabbé<sup>13</sup> showed that the glutathione quotient (GSH/R.B.C.) usually increased when the erythrocyte count was low. How much influence the common anemia of cancer patients had on the ratios is not apparent.

Parker and Kracke,<sup>14</sup> in the course of an experimental study of agranulocytosis, took up the torch for glutathione as the principal growth-stimulating factor. Their rabbits poisoned with benzol had decreased amounts of glutathione in the bone marrow and the blood. This was taken to indicate that the decreased cellularity of the blood and the marrow was due to a depletion by benzol of the normal acceleration factor for cell division, "which appears to be reduced glutathione." In a series of 14 patients with varied hematologic disorders they observed changes in the glutathione content of the blood. In 3 of the 4 instances of myeloid leucemia the reduced glutathione quotient (GSH/R.B.C.) was increased, while the absolute amount per 100 c.c. was decreased. In their conclusion they stated: "glutathione . . . showed a marked increase in those cases characterized by pathologic leukocytic stimulation; a marked decrease in those showing bone marrow aplasia, and with no change in those showing physiologic leukocytosis." They said further: "It is suggested that the reduced form of glutathione plays an important role in regulating normal bone marrow activity, and that depletion of this substance in the blood stream or bone marrow may lead to various leukopenic states." Their conclusions and interpretations are open to serious questioning on two scores. First, it does not seem proper to draw conclusions regarding granulopoiesis from the ratio GSH/R.B.C. This ratio, in fact, denies the significance of what glutathione may be present in leucocytes. Second, such conclusions accept the convictions of Hammett, Reimann and others, without any proof and make these convictions a basis for far-reaching deductions.

Respecting the reduced glutathione quotient, Gabbé showed that it was readily increased within two hours simply through producing an anemia by bleeding. Such a variable ratio seems to us to be a shaky foundation for a theory of pathogenesis. Moreover, other workers, as Pickard and Marsden,<sup>15</sup> concluded from their studies that: "there was no quantitative relation between the hemoglobin and the glutathione of the blood. Although the glutathione of the blood is a part of the contents of the erythrocytes its quantity does not follow variations in either the cell count or the cell volume. . . ." Senturia<sup>16</sup> who studied arthritics and found no significant alteration of the blood glutathione decided that: "In my opinion . . . none of these forms of expression (GSH:R.B.C. ratios and the like) contributed added information beyond the figures given for whole blood." Platt<sup>17</sup> examined, among other specimens, the blood from a patient with chronic myeloid leucemia, and the blood from one with chronic lymphoid leucemia. He separated the red and

TABLE IA  
(After Platt)

MYELOID LEUCEMIA		LYMPHOID LEUCEMIA
2,000,000	Erythrocytes	1,700,000
576,000	Leucocytes	200,000
82.5	Glutathione whole blood — mg per cent	19.6
5.0	Glutathione in 100 cc plasma — mg	6.0
72.0	Glutathione in 100 cc of erythrocytes — mg	71.0
151.0	Glutathione in 100 cc leucocytes — mg	67.0

TABLE IB

VARIATIONS OF THE REDUCED GLUTATHIONE OF THE BLOOD WHICH OCCUR WHEN SHED BLOOD IS LEFT STAND

CONTROL			AFTER 2 HOURS		
	FED GSII	TOTAL GSII		FED GSII	TOTAL GSII
	MC PER CENT			MC PER CENT	
1	51.0	42.0	25.7	40.5	
2	33.7	44.7	36.8	46.7	
3	30.7	39.9	24.5	38.0	
4	40.0	48.5	38.7	49.4	
5	37.7	44.8	34.1	43.9	

All values are averages of duplicate determinations

white blood cells, and analyzing them found the results that are reproduced in Table IA. Our own determinations of the GSII content of leucocytes yielded comparable values. Platt also analyzed 100 cc of pus, and found 164 mg of glutathione. These observations and many others, demonstrating the presence of glutathione in leucocytes, indicate that they should not be ignored in attempting to interpret variations of the blood glutathione. Finally, with respect to interpretation, it seems more desirable to us to attach greater significance to the "total glutathione" content than to the reduced glutathione content of the blood. The two are in some phase of equilibrium, and as we have already indicated, the reduced glutathione may be readily oxidized by atmospheric oxygen with the aid of such catalysts as copper, hemin and hemochromogens. Thus, if shed blood should be hemolyzed slightly or altered by contaminants in the collecting vessels the reduced glutathione concentration could change. Table IB illustrates the variation of the reduced glutathione which occurs when the blood sample is allowed to stand at room temperature for two hours before the filtrate is made. The total glutathione varies within the error of the method—about 10 to 15 mg. per cent. As contrasted with the variability of the reduced glutathione value, the total glutathione is estimated only after a zinc reduction of the filtrate, which eliminates all the oxidized glutathione (GSSG). Actually the value for glutathione obtained by iodate titration is too high by about 3 to 5 mg. per cent due to the presence of reduced ascorbic acid, thionine, methionine, etc. The error is fairly constant and is disregarded.

Our own study was undertaken to determine the significance, if any, of variations of the total glutathione content of the blood in blood dyscrasias. The technique used was that of Woodward and Fry.<sup>18</sup> Its accuracy was

TABLE II

DIAGNOSIS	PATIENT	NUMBER TESTS	REDUCED GLUTATHIONE MG. %	TOTAL GLUTATHIONE MG. %	NEWCOMER HB. GM. %	R.R.C.	W.B.C.	WINTROBE CELL VOL.
Normal	19	19	33.2 $\pm$ 1.30	42.16 $\pm$ 0.91	13.17 $\pm$ 0.221	4.970 $\pm$ 0.150	8.01 $\pm$ 0.561	43.2
Myeloid leucemia	8	14	32.38 $\pm$ 2.83	40.20 $\pm$ 2.58	10.11 $\pm$ 0.59	3.712 $\pm$ 0.295	75.84 $\pm$ 20.85	26.6
Pernicious anemia	12	20	33.92 $\pm$ 1.58	44.61 $\pm$ 1.50	15.31	4.749	7.23	40.7
Secondary anemia	7	8	27.92	40.48	12.62	4.206	9.16	37.1
Miscellaneous								
Banti's disease, hepatic cirrhosis	2	4	33.3	48.2	15.7	4.86	5.6	41.5
Erythroblastic anemia	1	4	22.9	29.8	9.8	4.72	10.6	34.0
Agranulocytosis	1	1	23.2	38.0	11.7	3.88	1.0	35.0
Polycythemia vera	2	6	65.36	75.16	23.5	8.43	12.2	69.0
	52	70						

checked by adding carefully weighed amounts of glutathione to whole blood. Recovery on five such experiments was 99, 101, 99.5, 100 and 102 per cent, respectively. Seventy-six assays for glutathione were made on the bloods of 52 patients who were seen by us in the medical and hematologic clinics of the University of Chicago. In addition to glutathione estimations the red blood cell count, white blood cell count, hemoglobin and cell volume values were all determined on the same sample. For the latter standard methods were employed. In Table II are listed the types of patients examined and the mean values with, in some instances the standard deviations of the results. Stru-  
ous but unsuccessful, efforts were made to effect some sort of correlation between the glutathione values and the red blood cell count, leucocyte count, hematocrit, and hemoglobin, and between various indices calculated from these determinations. That the glutathione content varied to a certain extent with the erythrocyte and leucocyte counts was obvious from inspection of some of the data where several estimations were made on individual patients. Examples of this visual relationship are presented in Table III.

TABLE III

DATE	FED CSH	TOTAL CSH	CELL VOLUME	HGB	RBC	WBC
<i>Patient 1</i> <i>Chronic Myeloid Leucemia</i>						
1/21	38.0	48.2	31	14.5	4.61	15,700
2/16	35.0	43.5	37	14.5	4.31	15,300
3/16	49.7	56.2	40	16.4	5.15	31,600
9/4	41.5	49.1	32	10.0	4.04	44,700
<i>Patient 2</i> <i>Chronic Myeloid Leucemia</i>						
1/14	55.0	62.5	21	10.1	3.22	110,000
1/30	30.0	43.0	28	8.5	2.19	28,700
2/16	21.7	32.0	25	9.8	3.93	4,200
<i>Patient 3</i> <i>Erythroblastic Anemia (Leucemia?)</i>						
1/19	23.3	27.3	35	10.7	5.40	11,300
2/9	19.7	29.5	41	9.8	4.60	—
2/15	20.5	28.5	35	9.6	4.51	9,200
2/16	28.0	32.8	28	9.5	4.40	11,400
<i>Patient 4</i> <i>Pernicious Anemia and Metrorrhagia</i>						
1/21	33.5	39.0	37	12.6	4.44	6,200
2/11	28.3	39.3	35	12.9	4.21	7,200
3/3	24.5	32.2	30	9.3	3.54	5,900
<i>Patient 5</i> <i>Polycythemia Vera</i>						
9/4	65.0	76.7	65	22.4	9.37	15,000
9/6	—	74.3	67	21.3	8.17	14,300
9/9	54.0	65.0	56	17.8	6.55	16,100

An effort at a tabular presentation of a possible influence of small variations in the leucocyte count was attempted. The result is shown in Table IV. All the values for total glutathione and hemoglobin were listed in seven categories of leucocyte counts regardless of diagnosis. The percentage variation of the average total GSH and the average hemoglobin of each category from the normal control mean value is interesting because of the almost complete lack of consistency.

By inspection of Tables III and IV it is apparent that there is some tendency for the glutathione of the blood to vary in the same direction as changes in the erythrocyte and leucocyte counts. This is not surprising, for

TABLE IV  
(A) Categories and Averages

	AVERAGE HB.—GM. %	AVERAGE TOTAL GSH MG. %	LEUCOCYTE RANGE
A	11.70	38.00	1,000- 2,500
B	12.63	42.60	2,600- 5,000
C	15.00	43.17	5,100- 7,500
D	14.46	41.86	7,600- 10,000
E	13.46	37.80	10,100- 15,000
F	9.96	38.92	15,100-100,000
G	10.78	52.44	over 100,000
Normal mean	15.17	42.16	8,010

(B) Percentage Variations of the Averages in (A) From the Normal Mean Values

CATEGORY	HB. PER CENT	TOTAL GSH PER CENT	NUMBER IN CATEGORY	W.B.C. PER CENT
A	-23	-10	1	- 87
B	-17	+ 1	7	- 34
C	- 1	+ 3	18	- 19
D	- 4	- 0.7	11	+ 12
E	-11	-10	9	+ 40
F	-34	- 8	8	+ 510
G	-22	+24	5	+1450

it is generally believed that the majority of the blood glutathione is in the cells. However, there is no evidence to indicate that the quantity in individual cells is constant. There is, further, no positive implication in any biochemical study which would suggest a causation for the variation that occurs.

#### CONCLUSIONS

1. From a study of blood samples from 52 patients, it appears that variations of the glutathione content in hematologic patients follow crudely changes in the number of formed elements in the blood.
2. When a group of normals are compared with a group of patients suffering from blood dyscrasias, the variations in the glutathione content of the blood are not statistically significant.
3. It is impossible for us to correlate changes in the blood glutathione with any variations of the erythrocyte and leucocyte counts, the hemoglobin, or the cell volume.
4. There is no good evidence which can be deduced from our data to justify any attempt to interpret the pathogenesis of blood dyscrasias on the basis of alterations of the blood glutathione.
5. The current views of the function of glutathione in the body's chemistry are reviewed, and the failure of modern biochemists to attribute to glutathione the role of "a universally occurring chemical stimulus to cell increase in number" is apparent.

#### REFERENCES

1. Lyman, C. M., and Barron, E. S. G.: Studies on Biological Oxidations. VIII. The Oxidation of Glutathione With Copper and Hemochromogens as Catalysts, *J. Biol. Chem.* 121: 275, 1937.
2. Hopkins, F. G., and Morgan, E. J.: Some Relations Between Ascorbic Acid and Glutathione, *Biochem. J.* 30: 1446, 1936.

- 3 Tait, H, and King, E J The Oxidation of Lecithin and Other Fatty Substances in the Presence of Glutathione, *Ibid* 30 285 1936
- 4 Meldrum, N U, and Tarr, H L A The Reduction of Glutathione by the Warburg Christian System, *Ibid* 29 108, 1935
- 5 Handovsky, H Ueber die Glutathion als Vermittler von Stoffwechsel und Tätigkeit, *Klin Wochenschr* 9 937, 1930
- 6 Gabbe, E Ueber Vorkommen und Bedeutung Leuchtlicher Schwefelverbindungen in den Blutkörperchen, *Ibid* 8 2077, 1929
- 7 Bumm, E, and Appel, H Ueber die Wirkung von Glutathion auf die Pasteursche Reaktion, *Ztschr f physiol Chem* 210 79 1932
- 8 Baker, Z Glutathione and the Pasteur Reaction *Biochem J* 31 980 1937
- 9 Haeper, W C Significance of Sulphydryl as a Growth Factor *Arch Path* 17 218, 1934
- 10 Hammett, F S Genetics, Sulphydryl and Cancer, *Science* 83 57 1936
- 11 Reimann, S P, and Hall, E M Protective Action of Sulphydryl Against Carcinogenesis Induced by 1 2 5 6 Dibenanthracene, *Arch Path* 22 50 1936
- 12 Schoonover, J W The Blood Glutathione in Human Cancer *Am J Cancer* 23 311 1935
- Idem The Plasma and Erythrocyte Glutathione in Human Cancer *Ibid* 23 315 1935
- 13 Gabbe, E Loc cit
- 14 Parker, F P, and Kricke, R R Further Studies in Experimental Granulopenia With Particular Reference to Sulphydryl Metabolism in Blood Disorders *Am J Clin Path* 6 41, 1936
- 15 Pickard, R J, and Marsden, C S The Relation of Blood Glutathione to Hemoglobin and the Amount of Red Cells, *J Lab & Clin Med* 19 205 1934
- 16 Senturia, B D Glutathione Content of Blood in Chronic Arthritis and Rheumatoid Conditions, *Ibid* 19 1151, 1934
- 17 Platt, R The Blood Glutathione in Disease *Brit J Exper Path* 12 139, 1931
- 18 Woodward, G E, and Fry, E G The Determination of Blood Glutathione, *J Biol Chem* 97 465, 1932

## THE "AVERAGE DAILY RETICULOCYTE RESPONSE" DURING THERAPY IN PERNICIOUS ANEMIA\*

ARNOLD FRIEDMAN, A B, RAPHALL ISAACS, M A M D AND ANNE LUFKIN, A B  
ANN ARBOR MICH

THE definite relationship between the level of the red blood cell count in patients with pernicious anemia and the subsequent reticulocyte content of the peripheral blood after therapy was first pointed out by Minot and his associates<sup>1</sup> Later Riddle<sup>2</sup> developed a formula for the correlation of the initial red blood cell count and the maximum reticulocyte percentage after oral liver extract therapy Bethell and Goldhamer<sup>3</sup> amplified this, adding data for desiccated stomach and liver extract intravenously Appreciating the fact that some patients did not reach the calculated peak of reticulocyte production, although the response was otherwise satisfactory and noting the different lengths of time required for the total "reticulocyte response," Bethell<sup>4</sup> developed a formula to correlate the "area" of the plotted reticulocyte curve with the initial red blood cell count This was used as an expression of the degree of bone marrow involvement Bethell's formula added the factor of total time, represented by the length of the base of the figure when the reticulocyte curve was plotted

\*From the Thomas Henry Simpson Memorial Institute for Medical Research University of Michigan Ann Arbor

Received for publication July 18 1938

TABLE I

The "Average Expected Daily Reticulocyte Percentage" After Treatment in Pernicious Anemia, With Various Initial Red Blood Counts Calculated From the Formula  $\frac{20.2 - 4.1 \text{ Eo}}{1 + 0.25 \text{ Eo}}$

Eo	"R"
0.4	16.9
0.5	16.1
0.6	15.4
0.7	14.7
0.8	14.1
0.9	13.4
1.0	12.9
1.1	12.3
1.2	11.7
1.3	11.2
1.4	10.7
1.5	10.2
1.6	9.7
1.7	9.3
1.8	8.8
1.9	8.4
2.0	8.0
2.1	7.6
2.2	7.2
2.3	6.8
2.4	6.5
2.5	6.1
2.6	5.8
2.7	5.4
2.8	5.1
2.9	4.8
3.0	4.5
3.1	4.2
3.2	3.9
3.3	3.7
3.4	3.4
3.5	3.1

Eo—Initial red blood count in millions per c. mm.

"R"—Average daily reticulocyte percentage.

In an effort to develop a simple relationship between the initial red blood cell count (and therefore by implication, the total number of megaloblasts stored in the bone marrow) and the length of time necessary for blood production to reach an equilibrium (expressed by the number of days after therapy was started, before the reticulocyte delivery reached a relationship comparable to that in health) we have made the following study. The data were taken from an analysis of the complete reticulocyte response of 21 patients with pernicious anemia treated with desiccated defatted stomach tissue, 22 patients treated with liver extract by mouth, and 39 patients treated with liver extract intramuscularly. The patients fulfilled all the requirements for the ideal study of reticulocyte reactions in pernicious anemia and were selected because of absence of complicating factors.

The reticulocyte percentage during the complete reticulocyte response, starting with the day after the treatment was started, and ending with the day (inclusive) on which the per cent fell to 3 or lower, were added. This number was then divided by the number of days represented. This "average daily reticulocyte per cent" is, of course, an arbitrary number, not represent-

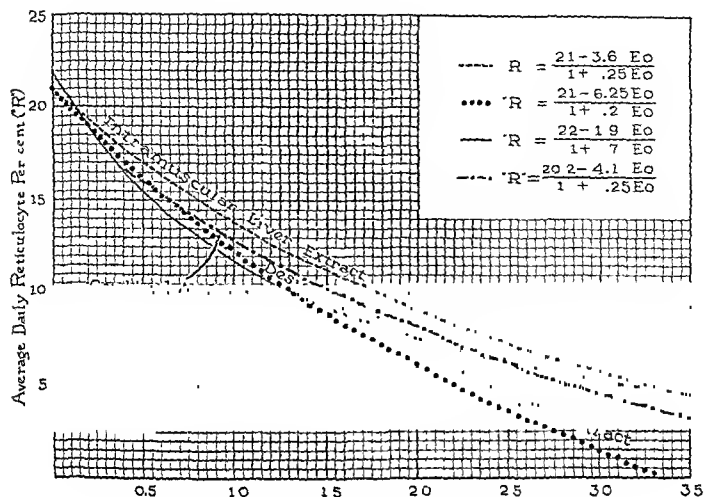


Fig. 1.—Initial red blood cell count in millions per cubic millimeter (Eo)

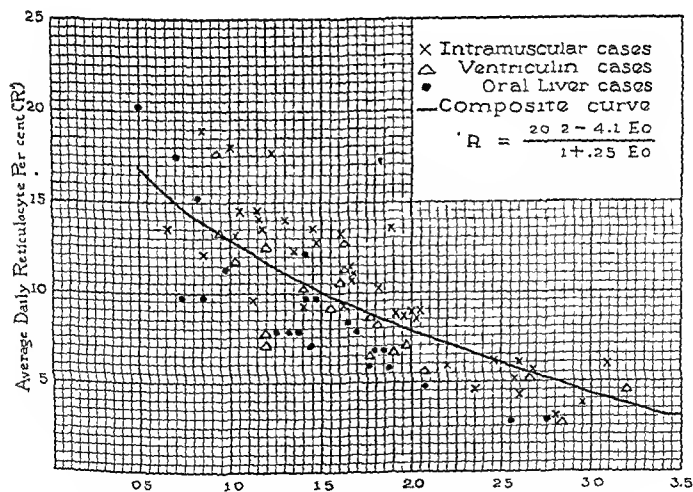


Fig. 2.—Initial red blood cell count in millions per cubic millimeter (Eo).



ing any actual observation, but serving as an index in which a number comparable to the "total" reticulocyte production is correlated with the time element in the process. These numbers were plotted against the initial red blood cell count, and a formula calculated for the curve.

From the data, the total number of days required to complete the "reticulocyte response" were plotted against the initial red blood cell count. There was no correlation evident. Similarly, there was no correlation between the initial red blood cell count and the day on which the maximum reticulocyte per cent was reached.

#### DISCUSSION

When the observed data of the "average daily reticulocyte per cent" are plotted separately, the trend for the cases treated with liver extract by mouth is slightly lower than that of those receiving desiccated stomach, and this, in turn, is not as high as that of the intramuscularly treated group (Fig. 1). In view of the dispersion of the plotted dots on the curve, it appears to be satisfactory to combine all of the cases in one group (Fig. 2). The formula for the curve which best fits the intramuscular group is:

$$\text{"Average daily reticulocyte per cent"} = \frac{21 - 3.6 E_0}{1 + 0.25 E_0}$$

For the desiccated stomach group:

$$\frac{22 - 1.9 E_0}{1 + 0.7 E_0}$$

For the oral liver extract group:

$$\frac{21 - 0.25 E_0}{1 + 0.2 E_0}$$

For the total group:

$$\frac{20.2 - 4.1 E_0}{1 + 0.25 E_0}$$

#### SUMMARY AND CONCLUSIONS

1. There appears to be a simple relationship between the level of the red blood cell count on the day that treatment is started in patients with pernicious anemia and the total reticulocyte production, as expressed by the total reticulocyte percentages of each day divided by the number of days required for the total "reticulocyte response."

2. The curve which best fits this relationship has a formula for each point of

$$\text{"Average reticulocyte per cent"} = \frac{20.2 - 4.1 E_0}{1 + 0.25 E_0}$$

3. This arbitrary relationship is an expression of the total reticulocyte production, and, therefore, a measure of the number of cells blocked at the megaloblast stage in the bone marrow of untreated patients with pernicious anemia.

4. There was no simple correlation between the total number of days required to complete the reticulocyte response and the initial red blood cell count.

5. There was no simple correlation between the initial red blood cell count and the day on which the maximum red blood cell count was reached.

## REFERENCES

- 1 Minot G R, Murphy, W P, and Stetson, R P Response of the Reticuloocytes to Liver Therapy, Particularly in Pernicious Anemia Am J M Sc 175 591 1928
- Minot, G R, Cohn, F J, Murphy, W P, and Lawson, H A Treatment of Pernicious Anemia With Liver Extract Effects Upon the Production of Immature and Mature Red Blood Cells, Am J M Sc 175 599, 1928
- 2 Riddle, M C Pernicious Anemia Blood Regeneration During Early Remission, Arch Int Med 46 417, 1930
- 3 Bethell, F H, and Goldhamer, S M Standards for Maximum Reticuloocyte Values Following Ventriculin and Intravenous Therapy in Pernicious Anemia, Am J M Sc 186 480, 1933
- 4 Bethell, F H The Relation Between the Total Reticuloocyte Production and the Degree of Bone Marrow Involvement in Pernicious Anemia, Am J M Sc 188 476, 1934

THE PHARMACOLOGIC ANTAGONISM OF METRAZOL AND SODIUM  
AMYTAL AS SEEN IN HUMAN INDIVIDUALS  
(SCHIZOPHRENIC PATIENTS)\*

LOUIS H COHEN, M.D., WORCESTER, MASS

THE antagonistic action of metrazol and the barbiturates has been the subject of considerable work by numerous investigators. The pioneer work of Tartler<sup>1</sup> demonstrated clearly that sodium barbital narcosis was quickly overcome by metrazol and that the convulsant threshold of metrazol was raised about fourfold by sodium barbital. These observations have been confirmed and extended by the work in animals of Mehl,<sup>10</sup> Malone,<sup>9</sup> Kohn and Jacobi,<sup>7</sup> Biehler,<sup>4</sup> Zipf and co workers,<sup>13, 14</sup> Amacher,<sup>2</sup> Albus,<sup>1</sup> Schwab and Jung,<sup>11</sup> Gros and Hofmann,<sup>5</sup> and Jackson.<sup>6</sup> The concept of the "awakening effect" (Koppanyi and associates<sup>8</sup>) has been applied by Bailow<sup>3</sup> to metrazol in explanation of its *modus operandi* in overcoming the effects of the barbiturates. To my knowledge there is no report in the literature concerning the effects in human individuals of the simultaneous injection of both of these drugs.

The experiments to be described here were primarily designed to test the psychiatric effects of metrazol when combined with sodium amytal. They have relevance to the recently introduced convulsive treatment of schizophrenia and other mental disorders with metrazol. Some of the data obtained seemed to be of sufficient pertinence to the pharmacologic problem of the barbiturate-metrazol antagonism to warrant separate report. The effects of rapid simultaneous, intravenous injection of combined metrazol and sodium amytal in a group of 5 schizophrenics were studied †

## PROCEDURE

Of the subjects studied, 4, M C, G L, W T, and L C, were women, and one, L O, was a male. All were chronically disturbed patients who were part of a large group which I treated for a period of fourteen to twenty three

\*From the Research Service of the Worcester State Hospital.

Received for publication August 8 1938

†It may be mentioned that sodium amytal in dosages sufficient to induce narcosis of greater or less duration is also used independently in the treatment of schizophrenia.

successive days with metrazol (Billhuber-Knoll). Metrazol in 10 per cent solution only was used. Two or three days following the cessation of this series, the following steps of the experiment were undertaken in order:

(1) The convulsive threshold of metrazol was redetermined.

(2) The following day and on successive days thereafter, the convulsive threshold dose of metrazol combined with 0.25 gm. sodium amytal (Lilly) was injected. The drugs were mixed together and injected intravenously as rapidly as possible. The amount of metrazol was increased daily until a convulsion occurred.

(3) On the day following the occurrence of the convulsion, 0.25 gm. sodium amytal was injected alone, over a period of two and one-half minutes.

(4) The following days and on successive days thereafter, metrazol combined with 0.5 gm. sodium amytal was injected under the same conditions as (2). The amount of metrazol was increased daily until a convulsion occurred.

(5) On the day following the occurrence of the convulsion, 0.5 gm. sodium amytal was injected alone over a period of two and one-half minutes.

In patient L. O., because of the high convulsant threshold obtained with metrazol combined with 0.25 gm. sodium amytal, the experiments with 0.50 gm. sodium amytal were not carried out.

#### RESULTS AND CONCLUSIONS

Since in most patients the dosage of metrazol alone must usually be increased after successive injections in order to produce convulsions, it is necessary to evaluate the data in the light of this factor of changing "tolerance." For each of the patients, therefore, a graph is presented of the dosages used and the convulsions produced over the period of from fourteen to twenty-three successive days, during which metrazol alone was administered. On the same graph there are presented the data on the dosages of combined metrazol-sodium amytal after the two-day or three-day interval of nonmedication. The points at which convulsions occurred are also indicated. The effects of the metrazol-sodium amytal mixtures and of sodium amytal alone are written on this portion of the graph in the appropriate places.

From the data in Fig. 1 the following points of significance may be noted:

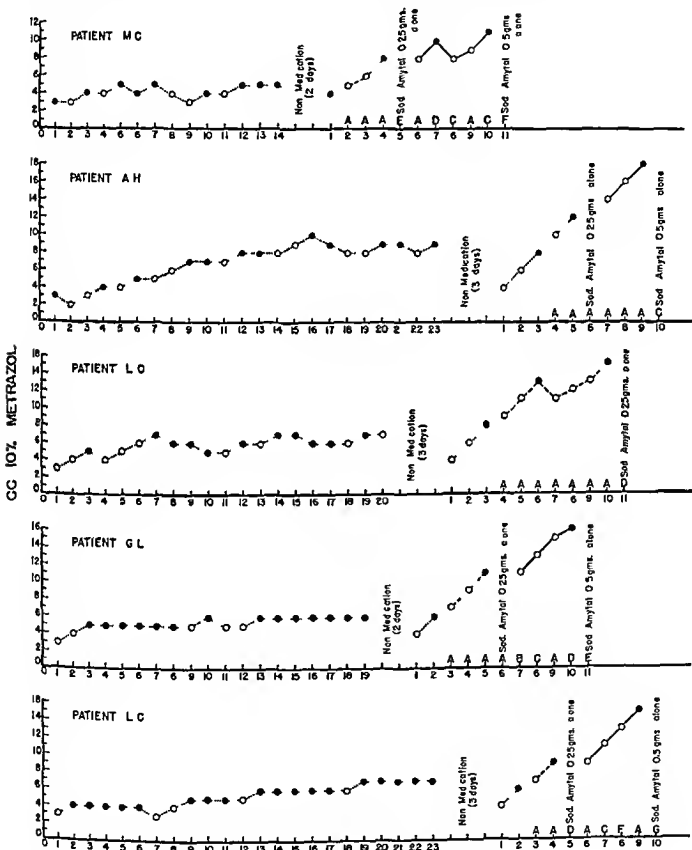
1. Certain combinations of sodium amytal and metrazol may be injected intravenously at a rapid rate in human individuals with no hypnotic or convulsive effects.

2. Sodium amytal raises the convulsive threshold of metrazol. Conversely, metrazol in convulsive dosages diminishes the hypnotic effect of sodium amytal, even when convulsions are not produced. The upward change in the convulsive threshold of metrazol, when combined with sodium amytal, is far greater than that of metrazol alone.

3. The convulsive threshold of metrazol seems to bear a direct relationship to the amount of sodium amytal with which it is combined. Whether or not the hypnotic effect of sodium amytal bears an inverse relationship to the amount of metrazol with which it is combined cannot be established from these data.

4 In patients in whom relatively small dosages of sodium amytal (0.25 gm intravenously) alone produce no apparent hypnotic effect, the same amount is effective in raising the convulsive threshold of metrazol

5 The inference seems clear that the antagonism of sodium amytal and metrazol (as indicated by the neutralization of the hypnotic effects of the



#### SUCCESSIVE DAYS

- Absence of convulsion  
 ● Occurrence of convulsion  
 Metrazol alone  
 Metrazol combined with 0.25 gm sodium amytal  
 Metrazol combined with 0.5 gm sodium amytal
- A—No drowsiness  
 I—Drowsy rest of day  
 C—Drowsy fifteen hours  
 D—Drowsy fifteen to twenty minutes  
 E—Slept twenty to thirty minutes followed by drowsiness  
 F—Slept one to two hours  
 G—Slept six hours

former and the convulsant effects of the latter) is one which may depend upon the action of both drugs on the same centers of the nervous system. There is also the presumption that the speed of action of both drugs must be very nearly the same.

## SUMMARY

The mutually antagonistic pharmacologic action of metrazol and sodium amytal has been studied in human individuals (schizophrenic patients) under conditions of rapid, simultaneous, intravenous injection of both drugs. The antagonism consists in the inhibition of the hypnotic effects of sodium amytal by metrazol and in the rise of the convulsant threshold of metrazol by sodium amytal. The efficacy of the antagonistic effect of sodium amytal to metrazol seems to depend upon the relative amounts administered. It is suggested that both drugs probably affect the same centers in the nervous system and at the same rate of speed.

## REFERENCES

1. Albus, G.: Tierexperimentelle Untersuchungen über die Weckwirkung handelsüblicher Analeptica unter besonderer Berücksichtigung von Cardiazol und Coramina, *Arch. f. exper. Path. u. Pharmacol.* 182: 471, 1936.
2. Axmacher, F.: Über erregende und analeptische Eigenschaften des Methylimidazols nebst Vergleich mit der Wirkung von Cardiazol und Coramin, *Arch. f. exper. Path. u. Pharmacol.* 183: 478, 1936.
3. Barlow, O. W.: The Effectiveness of Therapeutic Measures After Poisoning With Sublethal and Lethal Dosages of Barbitol in the Rabbit, *J. Lab. & Clin. Med.* 23: 601, 1938.
4. Biehler, W.: Krampfgrenze und Krampfhemmung, *Arch. f. exper. Path. u. Pharmacol.* 178: 693, 1935.
5. Gros, O., and Hofmann, H.: Über die Wirkung von Cardiazol, Ephedrin und der Kombination von Cardiazol und Ephedrin bei Veronalvergiftung, *Klin. Wchnschr.* 15: 1340, 1936.
6. Beeinflussung der Spättodesfälle, sowie der Gesamtmortalität durch Cardiazol, Ephedrin und der Kombination von Cardiazol und Ephedrin nach der letalen Gabe von Veronalmatrium, *Arch. f. exper. Path. u. Pharmacol.* 183: 138, 1936.
7. Jackson, D. E.: Variations in Reaction of Different Parts of the Central Nervous System as Influenced by Depressant and Stimulating Drugs, *J. Lab. & Clin. Med.* 20: 1, 1934.
8. Kohn, R., and Jacobi, M.: Untersuchungen über qualitative und quantitative Beziehungen zwischen Schlafmitteln und Analeptics, *Arch. f. exper. Path. u. Pharmacol.* 179: 448, 1935.
9. Koppányi, T., Linegar, C. R., and Dille, J. M.: Studies on Barbiturates: Analysis of Barbiturate-Picrotoxin Antagonism, *J. Pharmacol. & Exper. Therap.* 58: 199, 1936.
10. Maloney, A. H.: An Analysis of Stimulant-Depressant Action of Caffeine, Coramine, and Metrazol (Cardiazol) Based on Experimental Data, *Arch. internat. de pharmacodyn. et de therap.* 52: 373, 1936.
11. Contradictory Actions of Caffeine, Coramine, and Metrazol, *Quart. J. Exper. Physiol.* 25: 155, 1935.
12. Mehl, W.: Addition der Wirkung von einigen Analeptics zu Medinal, *Arch. f. exper. Path. u. Pharmacol.* 151: 41, 1930.
13. Schwab, R., and Jung, J.: Über Wirkung von chemischen Weckreizen bei Schlafmittelvergiftung des Meerschweinchens, *Ztschr. f. d. ges. exper. Med.* 99: 749, 1936.
14. Tartler, O. P.: Über den Antagonismus und Synergismus zwischen einigen Analeptics und Medinal, *Arch. f. exper. Path. u. Pharmacol.* 143: 65, 1929.
15. Zipf, K.: Kreislaufanaleptica als Weckmittel, *Arch. f. exper. Path. u. Pharmacol.* 181: 156, 1936.
16. Zur Entgiftung von Schlafmitteln und Basisnarkotica, *Arch. f. exper. Path. u. Pharmacol.* 181: 160, 1936.
17. Zipf, K., Windschus, W. A., and Kokosehka, F.: Zur Wirkung von Cardiazol, Coramin, Hexeton, Strychnin und Ikorol gegenüber Narkotica, *Arch. f. exper. Path. u. Pharmacol.* 185: 113, 1937.

## EXAMINATION OF EFFUSIONS IN TUMOR CASES\*

KURT E. LANDÉ, M.D., HAGLSTOWN, MD

RECENT experiences and doubts concerning the reliability and practical value of pathologic examinations of effusions in tumor cases prompted the following short survey.

During the first ten year period of a medium sized rural hospital the routine biopsies amounted to about 300, the number of autopsies to about 44 a year. Although the absolute figures have been gradually increasing within the last two years—1937, 512 biopsies, 43 autopsies; first six months of 1938, 209 biopsies, 45 autopsies—the above quoted averages of the last decade since 1928 still give a fairly accurate measure of the material available for investigation in the pathologic laboratory of the hospital.

An attempt was made in 27 cases either to corroborate or to exclude a tumor diagnosis and eventually to arrive at a conclusion about the cellular characteristics of a given growth through microscopic investigation of chest or abdominal effusions. The examination was carried out in chest fluid in 16 cases, in abdominal fluid in 8 cases, in one patient pleural transudate and ascitic fluid both were available. Once the stomach contents and once the fluid from a punctured cyst of the parotid gland were examined without success. On the whole, the tests have been carried out in 50 instances.

The findings for this survey were ascertained as follows. The available slides were studied, and a tentative diagnosis based upon an exact histologic description of the cellular peculiarities of the effusion, was made. All the clinical, autopsy, biopsy and operative notes available were collected and compared with the original pathologic description and diagnosis of the respective specimens. A comparison of the two diagnoses proved an almost complete conformity. An attempt to improve originally incorrect or doubtful diagnoses failed.

The procedure recommended originally by Mandlebaum<sup>1</sup> and later by Zemansky<sup>2</sup> was applied with only slight changes in minor details, partly similar to those mentioned and suggested by Foot<sup>3</sup> in 1937. Formalin and Zenker fixation gave identical results. Centrifugation and filtration of the fixed sediment seemed to be slightly superior to repeated centrifugations. It may well be that the less dense packing of the cellular elements in the sediment after only one single centrifugation results in a better dehydration under the treatment with alcohol. Paraffin sections of the sedimented cells were, in our experience, far superior to simple smears of sedimented material, fixed and treated in the fashion of the usual blood stain. Hematoxylin-eosin, eosin-methylene blue, and Wright's stain were of almost equal tinctorial value and sufficient for the purposes of differential diagnosis.

\*From the Mary Imogene Bassett Hospital, Cooperstown, N. Y.  
Received for publication August 13, 1938.

In reviewing the results of this small series of fairly representative material for judging the diagnostic procedure (see Table I) one recognizes that, as far as differential diagnosis is concerned, negative findings are apparently of less significance than positive results. The latter, marked by the presence of tumor cells in pleural or abdominal effusions, establish the diagnosis beyond any reasonable doubt, whereas in those cases in which the clinical diagnosis leans more towards a nonneoplastic origin of the effusions, the absence of tumor cells in the respective fluids will be only one more link in the whole chain of diagnostic evidence against the presence of a malignant growth. It is easily understood that the diagnostic problem is of especially great significance in those cases in which a pleural or abdominal effusion is caused by a systemic growth of the lymph glands, as, for instance, in Hodgkin's disease, lymphosarcoma, or generalized metastatic involvement of the lymph glands in certain rare cases of carcinoma of the stomach or prostatic gland. The chylous appearance of the fluids in some effusions may eventually add considerably to the uncertainty of the findings.

Two reasons are obvious for the difficulties encountered in the interpretation of the examinations. One rests on the fact that a good number of tumors, although spreading intraperitoneally or intrapleurally, do not give rise to the presence of tumor cells floating within or on the surface of the effusions. The very instructive descriptions and pictures of Quensel<sup>4</sup> discuss this factor in all the necessary detail and show that this possibility always has to be kept in mind whenever a negative histologic finding contradicts otherwise sufficient and striking clinical evidence in favor of a neoplasm.

The other factor is found in difficulties of correctly interpreting the cells encountered in pleural and abdominal effusions. It would lead too far afield to discuss all the possible sources of errors, starting with technical shortcomings; for instance, in consequence of delayed examination of the fluids, necrobiotic cellular changes may have taken place before adequate fixation. It seems to be unnecessary to dwell on these possibilities, since several authors rather recently have described the cellular findings in effusions with much detail, some of them adding excellent and instructive pictures; furthermore they have discussed the above-mentioned possible technical errors (Graham,<sup>5</sup> Quensel,<sup>6</sup> Karp,<sup>7</sup> Merklen and associates<sup>8</sup>). These papers all deal with the importance of correctly differentiating the plaques of more or less swollen or otherwise changed endothelial cells from tumor cells as they are found in practically every effusion of longer duration. But, whereas it seems to be comparatively easy to come to a conclusion concerning the endothelial origin, even of a multinucleated cell, in relying on the multitude of criteria available, it is still more than difficult and very often doubtful to diagnose positively a single tumor cell. Quensel,<sup>6</sup> MacCarthy,<sup>9, 10</sup> and Foot<sup>3</sup> stress the value of the so-called nucleo-nucleolar index, whereas Guttman and Halpern<sup>11</sup> emphatically deny the diagnostic value of this phenomenon. Although in the present small series it was not possible to carry out correct measurements of the nucleo-nucleolar index, special attention has been paid to the presence of large nucleoli in cells, otherwise suggestive of neoplastic origin. But it was possible in only 2 out of 5 tumor cases with peritoneal or pleural spread, in which tumor cells could be expected and had not been found previ-

TABLE I

CASE NO	NO OF EXAM	DIAGNOSIS	TUMOR SPREAD			EFFUSION		TUMOR CELLS		
			+	?		AB DOMINAL	PLEURAL	+	?	-
2	5	Cystadenoma of ovary with metastases	+			+	+	+		
3	1	Endothelioma of pleura	+				+	+		
10	1	Cystadenocarcinoma of ovary	+			+		+		
25	3	Cystadenoma of ovary with metastases	+			+		+		
26	1	Carcinoma of breast with invasion of pleura	+				+	+		
22	2	Adenocarcinoma of pleura	+				+	+		
1	18		6				+	6		
1	4	Carcinoma of breast with metastases	+				+		0	
1	1	Small cell carcinoma of ovary	+			+			0	
8	1	Papillary carcinoma of ovary	+						0	
11	2	Adenocarcinoma of bronchus		?			+			1
19	1	Carcinoma of lung hemithorax		?			+			-
20	1	Secondary carcinomatosis of peritoneum		?						
21	1	Undiagnosed malignancy of kidney		?			+		?	
9	2	Hodgkin's disease or Ewing's sarcoma					+			
12	1	Neoplasma of mediastinum					+			
15	1	Adenocarcinoma of colon				+				
18	1	Primary carcinoma of liver				+				
27	4	Carcinoma of prostate with metastases to lymph glands					+			
11	2	Adenocarcinoma of head of pancreas		?		stomach				
10	16		1	6		3	6		1	9
4	1	Unspecific pleuritis					+			
6	2	Syphilitic heart disease					+			
7	1	Chronic pulmonary tuberculosis with pleurisy					+			
12	1	Arteriosclerotic heart disease				+				
14	2	Undiagnosed disease of lungs (retinomyces?)					+			
17	1	Cyst of parotid gland					cyst			-
23	1	Perforated ulcer of stomach, peritonitis				+				
24	1	Arteriosclerotic heart disease					+			
8	10					2	5			8
27	50	Summary								
		Tumors with spread 9+, 4?	9	4	6	10	11	6	?	18
		Tumors without spread 6								
		Other causes 8								

ously, to establish (retrospectively) the diagnosis on account of the presence of very large nucleoli. It may be that a correct micrometry of a wealth of material perhaps gives with increasing experience a greater feeling of security in the interpretation and evaluation of this special criterion. So far, however, it seems somewhat audacious to arrive at a positive tumor diagnosis in an otherwise doubtful specimen only on account of one definite cellular characteristic, namely, the very large nucleolus. Since multinucleation, mitoses, giant cavities signet ring formation, and tinctorial anomalies may be observed as well within cells of endothelial origin, the most valuable criterion is still the presence of typical tumor elements, not in the form of single cells, but of cell clusters acini or other larger histologic entities, as used in the common pathologic routine diagnosis of tissues.



It may be added as completion of Table I, that the diagnosis could be verified both by autopsy and by biopsy and operation nine times. Only in those 4 tumor cases where the metastatic spread to pleura or peritoneum is noted as questionable (Nos. 16, 19, 20, and 21 of Table I), the final proof for the correctness of the diagnosis was missing. The same holds true for 5 out of the 8 non-neoplastic cases.

A glance at Table I shows that in applying the above-mentioned criteria the diagnosis could be established at once in 6 out of 9 patients in whom the kind of pleural or peritoneal spread made the presence of floating tumor particles or cells more than probable. In 2 of the remaining 3 cases, the interpretation of the cellular findings was doubtful, but here, as already pointed out, the presence of large nucleoli, and probably even better the determination of the nucleo-nucleolar index, should have pointed in the direction of the correct tumor diagnosis. To these 3 negative, or more exactly questionable, results must be added 5 more cases, in which a pleural or peritoneal infiltration was very likely, although no autopsy findings were available to corroborate the diagnosis. Within the effusions of these cases no tumor cells could be discovered either. However, in one the verification of cells suggestive of neoplastic origin was at least suspected and jotted down as questionable, but not sufficient for a definite diagnosis (Case 21). On the other hand, in no case was a tumor diagnosis made where later at autopsy or operation it was conclusively shown that the presence of malignant cells within the fluid would have been highly improbable, if not impossible, on account of the absence of a peritoneal or pleural neoplastic involvement. In taking into account these cases and in adding others, where a nonspecific pleurisy or an ascites in a case of cardiac cirrhosis gave rise to the effusion, which likewise showed negative results, one sees that the diagnostic value of the method is not as small as first assumed. Although, naturally, negative findings are, as pointed out before, of far less diagnostic significance for the clinician, the danger of wrongly interpreting the abnormal cells of endothelial provenience as neoplastic cells is apparently negligible.

Two cases out of the foregoing series may be described briefly because of post-mortem findings which were of special interest.

No. 27, J. St., was a male patient, 48 years of age, who came to the hospital after a half year's illness. He had first noticed a swelling of his left leg which, in the course of the next few months, became gradually cyanotic and dyspneic. On admission a huge mediastinal mass and also a generalized swelling of his inguinal, cervical, and axillary lymph glands was discovered as cause of his symptoms. At the time of his sudden, rather unexpected death, the clinical diagnosis was lymphosarcoma or Hodgkin's disease. The possibility of a primary carcinoma of unknown origin with metastatic spread to the lymph glands was also discussed. At no time were any malignant cells found in the pleuritic effusion, although the endothelial cells, which were present in great numbers, single and in plaques, were first difficult to differentiate. At autopsy the presence of large masses of firm, yellowish gray lymph glands at the above-mentioned locations seemed to be in favor of a primary malignant disease of the lymph glands. The fact that the neoplastic masses in the pelvis invaded the seminal vesicles was noticed, but did not change the preliminary, gross diagnosis. Microscopic examination, however, revealed that not a primary systemic process of the lymph glands caused the growth, but a metastatic invasion originating in the prostatic gland. This kind of tumor spread, limited to the lymph glands, is known but very rare.

No 5, V. H., was a 47-year old married woman, who died eight and one half months after the onset of vaginal bleeding caused by a malignant tumor of the ovary. In spite of profuse spread through the peritoneal and pleural cavities, it was not possible to trace tumor cells within the abdominal effusion. Histologically, the tumor turned out to be a so called small cell carcinoma of the ovary. Among many metastases found at necropsy and microscopic study, one was encountered within the mitral valve. A careful search of the literature did not disclose the description of a similar location of a tumor metastasis.

#### SUMMARY

1. Pathologic examination of pleural and abdominal effusions in tumor cases has been tried out routinely in the material of a medium-sized rural hospital.

2. Attention is called to the fact that even in the presence of an intraperitoneal or intrapleural tumor spread, malignant cells within an effusion cannot be expected in every case, their occurrence depending upon the special conditions of the neoplastic growth.

3. The difficulties arising from the interpretation of the cellular findings and the necessary differential diagnosis between cells of endothelial and neoplastic origin are briefly discussed.

4. The number of cases in which tumor cells could be demonstrated was comparatively small in this series. However, the absence of any misinterpretation of endothelial cells as tumor cells, gives a somewhat increased diagnostic value to the negative findings.

5. One case of prostatic carcinoma with metastatic spread exclusively to the lymphoglandular system, and one of an ovarian carcinoma with a metastasis within the mitral valve, are briefly described.

#### REFERENCES

1. Mandlebaum, F. S. The Diagnosis of Malignant Tumors by Paraffin Sections of Centrifuged Exudates, *J. Lab & Clin Med* 2: 580, 1917.
2. Zemansky, A. Philip. The Examination of Fluids for Tumor Cells, *Am J M Sc* 175: 489, 1928.
3. Foot, Nathan C. The Identification of Tumor Cells in Sediments of Serous Effusions, *Am J Path.* 13: 1, 1937.
4. Quensel, Ulrik. Zur Frage der Zytodiagnostik der Ergüsse seröser Höhlen, *Acta med Scandinav* 68: 427, 1928.
5. Graham, George S. The Cancer Cells of Serous Effusions, *Am J Path* 9: 701, 1933.
6. Quensel, Ulrik. Zytologische Untersuchungen von Ergüssen der Brust und Bauchhöhlen mit besonderer Berücksichtigung der karzinomatösen Exsudate, *Acta med Scandinav* 68: 458, 1928.
7. Karp, Heribert. Cytodiagnostik maligner Tumoren aus Punktionen und Sekreten, *Ztschr f. Krebsforsch* 36: 579, 1932.
8. Merklen, Prosper, Witz, R., and Kabaker, J. Sur la cytologie des épanchements pleuraux, *Presse med* 41: 1828, 1933.
9. MacCarthy, William C., and Haumeder, Eva. Has the Cancer Cell Any Differential Characteristics? *Am. J Cancer* 20: 403, 1934.
10. MacCarthy, William C. The Value of the Macronucleolus in the Cancer Problem, *Am J. Cancer* 26: 529, 1936.
11. Guttman, Paul H., and Halpern, Sol. Nuclear Nucleolar Volume Ratio in Cancer, *Am J. Cancer* 25: 802, 1935.

# THE CONCENTRATION OF ACACIA IN THE SERUM, ITS RATE OF EXCRETION, AND ITS EFFECT ON THE COLLOID OSMOTIC PRESSURE FOLLOWING INTRAVENOUS INJECTION IN CASES OF CIRRHOSIS OF THE LIVER\*

HUGH R. BUTT, M.D., AND MARSCHELLE H. POWER, PH.D., ROCHESTER, MINN.  
AND ANCEL KEYS, PH.D., MINNEAPOLIS, MINN.

THE widespread use of acacia as a substitute for blood has focused considerable attention on this substance and its probable physical and chemical actions in the organism. The purpose of this study was to determine the concentration of acacia in the blood, its rate of excretion, and its effect on the colloid osmotic pressure following intravenous injection into man.

It has been established recently that acacia cannot be readily metabolized by the animal body, but that it is deposited in the liver, spleen, and other organs following intravenous administration (Andersch and Gibson,<sup>1</sup> 1934). Others have presumed that deposition of this substance in the liver accounts for the decrease in the levels of plasma protein sometimes observed following the intravenous administration of this agent (Dick, Warweg, and Andersch,<sup>2</sup> 1935).

Acacia disappears rather rapidly from the blood stream, but Keith, Power, and Wakefield<sup>3</sup> (1935) have been able to detect traces of acacia in the blood serum of man three years after its injection. There has been a lack of adequate data on the rate of disappearance of acacia following intravenous injection, and the data of Gasser, Erlanger, and Meek<sup>4</sup> (1919) on rabbits, of Huffman<sup>5</sup> (1929) on man, and of Peoples and Phatak<sup>6</sup> (1935) on dogs, are about all that are available.

## METHODS

The colloid osmotic pressure, the values for the serum proteins (albumin and globulin), and the rate of excretion of the injected acacia were determined in three cases of cirrhosis of the liver.

The concentration of acacia in serum and urine was determined by a precipitation method which has been described in preliminary form by Power<sup>7</sup> (1937). The details of the method will be published elsewhere. For serum the procedure involves, first, removal of proteins by means of trichloroacetic acid, and, second, isolation of acacia from the protein-free filtrate by means of acetone. The acidity of the serum filtrate should be equivalent to about 8.5 per cent trichloroacetic acid. Five cubic centimeters of acetone is mixed with 2 c.c. of filtrate in a moderately pointed centrifuge tube, and the mixture is allowed to stand an hour or more in the refrigerator. The precipitate of acacia is separated in the centrifuge, washed with acetone, and dried. It is then

\*From the Division of Medicine, the Mayo Clinic, the Division of Biochemistry, the Mayo Foundation, and the University of Minnesota, Minneapolis.

Received for publication, August 29, 1938.

oxidized with a known amount of potassium dichromate in the presence of concentrated sulfuric acid, after which the excess dichromate is determined iodometrically. It has been found that purified acacia that has been isolated and treated in this manner requires, on the average, 2.72 c.c. of 0.05 normal solution of dichromate per milligram for complete oxidation. The results of analyses may be calculated with the aid of this factor.

Acacia, as determined by this method, obviously represents a substance which has not become so modified in the body as to lose its property of precipitating in the presence of acetone.<sup>6</sup> If the acacia hydrolyzes to some extent, the simpler molecules formed might be partly metabolized or partly excreted in the urine. Qualitative experiments have indicated, in fact, that subsequent to intravenous injection of a solution of acacia, the urine may contain more furfural-yielding substances than can be accounted for by the amount of "acetone precipitable" acacia present. It may become of considerable interest to compare the excretion of "precipitable" acacia with that of total furfural-yielding substances in future studies of this sort.

The acacia employed in each case was a 6 per cent (with 0.9 normal sodium chloride) solution that had been sterilized in the autoclave. Mention is made that these solutions were autoclaved because this process supposedly increased the colloid osmotic pressure of the solution (Dodds and Haines,<sup>8</sup> 1934). The colloid osmotic pressure of the blood serum was measured by a modification of the membrane bag arrangement of Starling<sup>9</sup> (1896), Adam<sup>10</sup> (1928), Keys and Taylor<sup>11</sup> (1935), and others. The details of the method will be presented elsewhere, but a few essentials may be given here. The serum is diluted with an equal volume of a phosphate buffer solution at pH 7.4, which is approximately isoionic and isoosmotic with the serum. Small, rather rigid celloidion sacs, which have been previously tested and proved to be protein tight, are used. The same buffer solution is used for the external medium and the entire system is kept at 0° C. Usually, equilibration is complete within forty-eight hours, but in all observations in the present series the osmometers were observed for some days longer. At the end of equilibration the amount of nitrogen in both the inner and outer fluids was determined and the result compared with the values for the protein and nonprotein nitrogen of the original serum. The values for colloid osmotic pressure were corrected to the original concentration of the proteins in the serum by means of standard protein dilution curves. All determinations were made in duplicate. The mean colloid osmotic pressure of the serum obtained from thirty normal persons was found to be 371.3 mm. of water, with a probable error of  $\pm 5.38$ . The variability, as measured by the standard deviation, was 43.7 mm. of water.

## RESULTS

Following intravenous injection of a 6 per cent solution of acacia, the colloid osmotic pressure of the blood serum is assumed usually to be elevated only for forty-eight hours (Amberson,<sup>12</sup> 1937). Fig. 1 adequately supports this statement. It is interesting that the value for the total protein of the

\*It might be well to mention that the presence of acacia interferes in determinations of serum sulfate performed by benzidine methods in which acetone or alcohol is used as the precipitation medium (Keith, Power, and Wakefield, 1935).

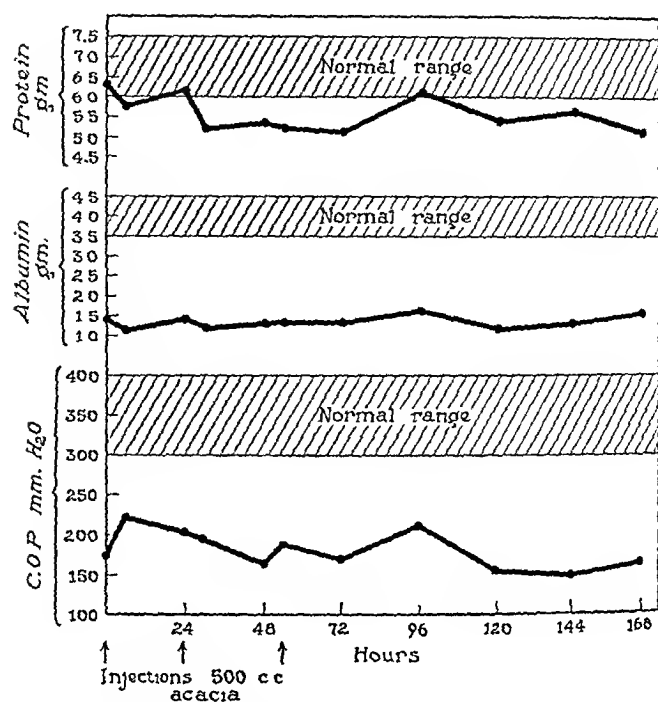


Fig. 1.—Effect of multiple injections of acacia on the colloid osmotic pressure and on the concentration of serum protein of man.

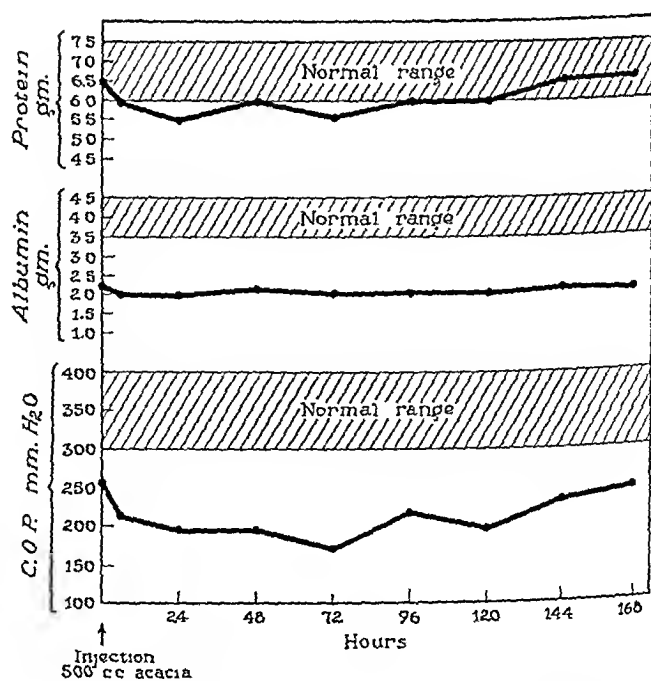


Fig. 2.—Effect of single injection of acacia on the colloid osmotic pressure and on the concentration of serum protein of man.

blood serum was diminished following injections of acacia, but that the albumin fraction was affected little. Similar results have been recorded by Heckel<sup>13</sup> (1938) and his associates, following large injections of acacia into dogs.

In the case summarized in Fig 2 there was only one injection of 500 cc of acacia. The colloid osmotic pressure of the blood serum in this instance was not maintained above, or at, the basal level of the patient for any appreciable period of time.

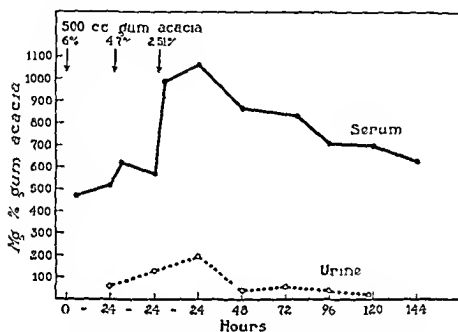


Fig 3—Concentration of acacia in serum and urine following multiple injections of acacia

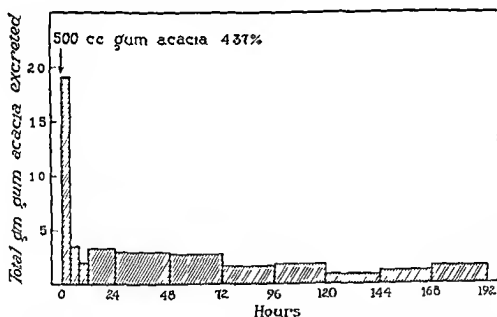


Fig 4—Urinary excretion of acacia following single injection of acacia

It is frequently stated in the literature that the serum proteins particularly the albumin fraction, fall to a low concentration in man and animals following the intravenous injection of solution of acacia (Dick and others, 1935, Heckel, 1938). Only in the third case, similar otherwise to the case represented in Fig 1, did the concentration of albumin decrease appreciably following this procedure, but at no time was this reduction of the magnitude reported in the literature.

Fig 3 represents graphically the concentration of acacia in the blood and urine of a patient with cirrhosis of the liver, following the intravenous injection of acacia. There appears to be some cumulative effect following the third

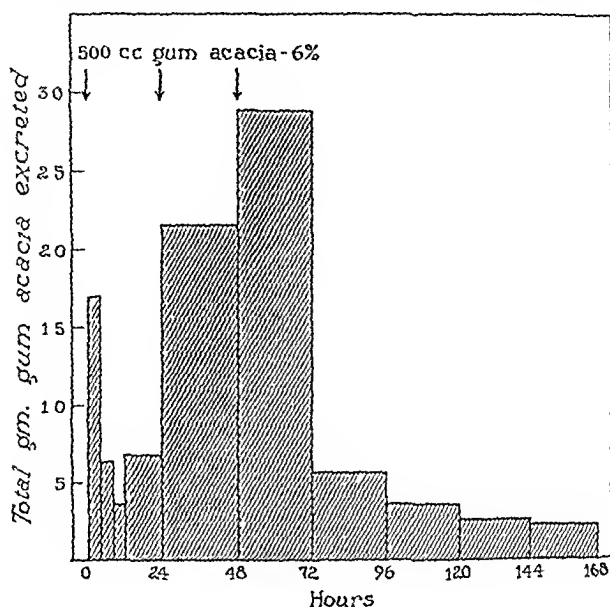


Fig. 5.—Urinary excretion of acacia following multiple injection of acacia.

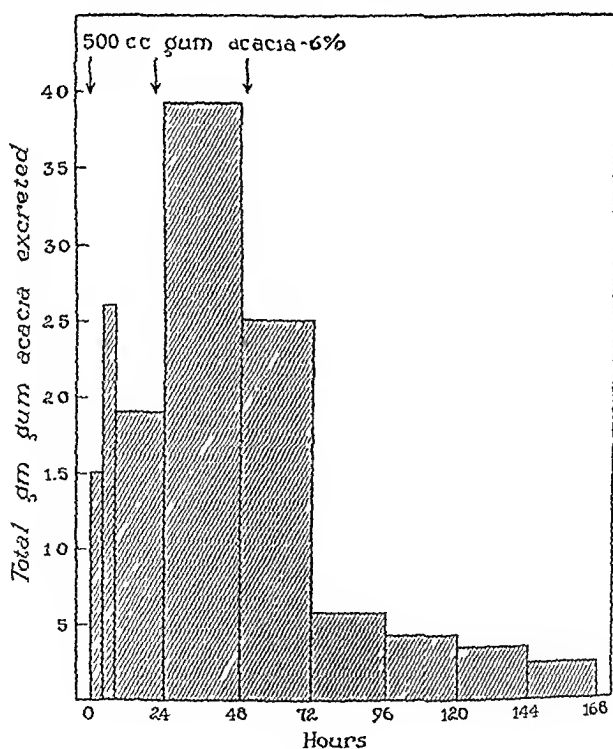


Fig. 6—Urinary excretion of acacia following multiple injection of acacia.

injection of acacia. The colloid osmotic pressure of the serum does not remain elevated in proportion to the concentration of acacia in the blood, probably because the smaller particles of acacia are excreted rapidly in the urine or diffuse rapidly into the tissues or some other depot.

In Figs 4, 5, and 6 the excretion of acacia in urine following the injection of various quantities of acacia is shown graphically. It will be seen that, for the period of time included in the graphs, the quantity of acacia excreted by the two patients who received multiple injections was in each case less than 20 per cent of the total amount of acacia injected, and that most of the acacia excreted appeared in the first three days following its administration. The patient who received only one injection excreted somewhat more than 20 per cent of the quantity injected during the period of observation. The acacia excreted in the urine probably is composed mainly of the smaller particles which have passed through the membrane of the glomerulus. This loss of osmotically active particles in the urine, together with reduction of the quantity of acacia in the serum as a consequence of other means of disposal in the body, such as storage, probably accounts for the inability of injections of acacia to maintain an elevated colloid osmotic pressure for any long period of time.

#### SUMMARY

Administration of acacia to man under the conditions of these observations results in a short temporary rise in the colloid osmotic pressure of the serum. The concentration of acacia in the serum remains high for a much longer period of time than does the colloid osmotic pressure. This discrepancy probably is the result of the rapid loss of osmotically active particles of acacia through the urine, or into the tissues or some other depot.

#### REFERENCES

1. Andersch, Marie, and Gibson, R. B. Studies on the Effects of Intravenous Injections of Colloids. I. Deposition of Acacia in the Liver and Other Organs and Its Excretion in Urine and Bile, *J. Pharmacol. & Exper. Therap.* 52: 200, 1934.
2. Dick, M. W., Warweg, Edna, and Andersch, Marie. Acacia in the Treatment of Nephrosis, *J. A. M. A.* 105: 674, 1935.
3. Keith, N. M., Power, M. H., and Wakefield, E. G. The Detection and Persistence of Acacia in the Blood, *Proc. Staff Meet., Mayo Clin.* 10: 38, 1935.
4. Gasser, H. S., Erlanger, Joseph, and Meel, W. J. Studies in Secondary Traumatic Shock. IV. The Blood Volume Changes and the Effect of Gum Acacia on Their Development, *Am. J. Physiol.* 50: 31, 1919.
5. Huffman, L. D. Solution of Acacia and Sodium Chloride in Hemorrhage and Shock. Effects of Intravenous Administration, *J. A. M. A.* 93: 1698, 1929.
6. Peoples, S. A., and Phatak, N. M. Spectroscopic Determination of Gum Acacia in Blood. Rate of Disappearance in Normal Dogs, *Proc. Soc. Exper. Biol. & Med.* 32: 635, 1935.
7. Power, M. H. A Volumetric Method for the Determination of Acacia in Serum, Lymph and Urine, *J. Biol. Chem.* 119: 77, 1937.
8. Dodds, E. C., and Haines, R. T. M. Osmotic Pressure of Gum Acacia Solutions. *Biochem. J.* 28: 499, 1934.
9. Starling, E. H. On the Absorption of Fluids From the Connective Tissue Spaces, *J. Physiol.* 19: 312, 1896.
10. Adair, G. S. A Theory of Partial Osmotic Pressures and Membrane Equilibria, With Special Reference to the Application of Dilton's Law to Hemoglobin Solutions in the Presence of Salts, *Proc. Roy. Soc., London* S. B. 120: 573, 1928.
11. Keys, Ancel, and Taylor, Henry. The Determination of the Colloidal Osmotic Pressure in Blood Serum and Similar Fluids, *J. Biol. Chem.* 109: 47, 1935.
12. Amberson, W. R. Blood Substitutes, *Biol. Rev.* 12: 48, 1937.
13. Heckel, G. P., Erickson, C. C., Yuile, C. L., and Knuth, R. E. Blood Plasma Proteins as Influenced by Intravenous Injection of Gum Acacia, *J. Exper. Med.* 67: 345, 1935.



## RAPID CONVERSION OF TRYPTOPHAN TO INDOL BY RESTING BACTERIUM COLI\*

STUART L. VAUGHAN, M.D., PH.D., BUFFALO, N. Y.

IN PREVIOUS communications I<sup>1, 2</sup> have shown that fresh, uncontaminated urine of the majority of human subjects contains an indol precursor which in all probability is free tryptophan, but no indol as such. This work, which has been confirmed by Carnes and Lewis,<sup>3</sup> suggests an approach to the study of free tryptophan in urine and other substances. Such study in the past has been hampered by the lack of sufficiently sensitive tests as well as by the difficulty in distinguishing free tryptophan from that in a combined state. By utilizing the principle employed in the experiments mentioned above, it should be possible to avoid these difficulties.

The principle combines selective conversion of free tryptophan into indol and subsequent analysis of the end product. The conversion is brought about biologically by the activity of bacteria. As previously carried out, this involved the growth of *Bacterium coli* in the test substances. Certain obvious disadvantages are inherent in such a method, and the present work was done in the attempt to avoid some of them by adapting to this purpose a "resting" bacterial suspension which resembles a chemical reagent rather closely.

Woods,<sup>4, 5</sup> using a suspension of washed *Bacterium coli*, was able to recover from tryptophan solution nearly 100 per cent of the theoretical yield of indol. The washed growth from 20 Roux bottle cultures was suspended in 20 e.e. of Ringer's solution and added to 100 e.e. of 0.02 per cent tryptophan solution in phosphate buffer of pH 7.2. Incubation was at 37° C., with continuous aeration. Indol was recovered very shortly after the incubation was begun, but complete conversion required from sixteen to thirty-six hours. He studied the action of suspensions on various indol derivatives also, and found that only one,  $\beta$ -indol pyruvic acid, was capable of being converted into indol, and that in small amounts under special circumstances.

Happold and Hoyle,<sup>6</sup> using a technique somewhat similar to that of Woods, obtained essentially the same results. These authors were able also to prepare killed suspensions of the bacteria that were almost equal in conversion potency to the unkilld. In their studies conversion required up to forty-eight hours.

The results which I obtained substantiate the essential findings of the above-mentioned works, and show further how a modified technique not only increases the speed of the reaction to a point where it is almost instantaneous, but also simplifies the procedure in such a way that it is more readily available for certain purposes. The results show also a possible fallacy in the assumption of Woods that *Bacterium coli* does not decompose indol.

\*From the Buffalo General Hospital and the University of Buffalo Medical School.  
Received for publication, August 30, 1938.

## METHODS

In general, the technique employed was that used by Quastel<sup>7</sup> and, more particularly, that used by Vaughan and Hubbard<sup>8</sup> in studies of the destruction of reducing sugars.

The stock strain of *Bacterium coli* known to produce indol actively was grown for twenty-four hours on beef juice agar slants in tubes 1 inch in diameter. The growth was removed with a small volume of physiologic saline and washed repeatedly in saline by centrifuging until the washings gave a negative test for indol. The second washing always gave a negative test, but the organisms were washed three times as an added precaution. Before the final washing, the suspension was divided among a series of tubes in such a way that an appropriate volume of packed sediment would be obtained (usually 0.2 c.c.). After centrifuging, the supernatant liquid was drained off and the tubes containing the bacterial sediment were ready to receive the test fluid. This was added in 3 c.c. quantities and the sediment evenly suspended in it. After a suitable incubation period, the tubes were centrifuged again and the supernatant liquids transferred to small agglutination tubes to be tested for indol as follows: To the 3 c.c. of test liquid were added 1.5 c.c. of petroleum ether. The tube was inverted fifty times to insure uniform extraction of indol. One cubic centimeter of the clear ether layer was then transferred to another tube, and 0.5 c.c. of Ehrlich's reagent was added. The contents were agitated by tapping the tube with the finger until the maximum color was produced in the ether layer.

## EXPERIMENTS

I. The first experiment was an attempt to convert tryptophan into indol rapidly when the former substance was dissolved in substrates favorable for bacterial growth. For this purpose the following solutions were employed: (a) tryptophan broth, (b) peptone water, (c) meat juice broth containing the usual amount of peptone, (d) urine, and (e) ascites fluid. A liberal amount of bacterial sediment was added (0.5 c.c.), and the tubes were incubated for twenty minutes at 37° C. In each instance a positive test was obtained.

II. In the second experiment solutions of tryptophan that failed to support a vigorous growth of the organisms were examined. Varying dilutions of tryptophan in distilled water were tested in duplicate. One set was seeded with the organism as in the usual cultural technique, and incubated for forty-eight hours at 37° C. Tests for indol were uniformly negative. The other set was tested in the manner described in the first experiment. Indol was detected in dilutions from 1:1,000 to 1:2,000,000. When the tryptophan was dissolved in physiologic saline and tested in the same way, indol could be detected irregularly only by the cultural technique, while it could be demonstrated in high dilutions by the "resting" bacteria method.

III. The third experiment was a rough attempt to analyze a few of the many factors entering into the reaction.

A. Time: Dilutions of tryptophan from 1:1,000 to 1:3,000,000 in distilled water were treated with the bacterial sediment in duplicate. Incubation was

at room temperature. The first set was incubated for five minutes. The highest dilution giving a color reaction was 1:200,000. The second set was incubated for twenty minutes. The positive test was obtained in a dilution of 1:1,000,000.

B. Temperature: The time of incubation was constant (five minutes). Three temperatures, 0° C., 20° C., and 37° C., were used. All operations, including centrifugation, were carried out as nearly as possible at the given temperature. The results were as follows:

0° C.	Negative tests in all dilutions
20° C.	Positive test in dilution of 1:200,000
37° C.	Positive test in dilution of 1:1,000,000

C. Volume of Bacterial Sediment: A tryptophan dilution of 1:100,000 in physiologic saline was incubated for five minutes at 37° C. in tubes containing varying amounts of bacterial sediment and examined for intensity of color produced in the indol test. The maximum color was produced with any amount of sediment from 0.2 c.c. upwards. Using 0.1 c.c., the color was very faint. Prolonged incubation with such small volume increased the color intensity somewhat.

D. Effect of Glucose: As shown by Logie,<sup>9</sup> the amount of free indol found in a peptone water culture of *Bacterium coli* is diminished if 1 per cent of glucose is added before inoculation. The same result was obtained in the present experiment with "resting" organisms. To one of two tubes of 1:100,000 tryptophan in physiologic saline was added 1 per cent of glucose. Both solutions were treated with bacterial sediment for five minutes at 37° C. The maximum color developed in the indol test upon the solution to which no glucose was added. With the glucose solution a very faint, though distinct, color resulted.

IV. The fourth experiment was an attempt to quantitate the amount of indol produced.

A. Colorimetric comparison between the intensity of the reaction in the test solution and that of solutions of pure indol: On the basis of the work of Logie<sup>9</sup> that indol is utilized to some extent by the bacteria, it seemed probable that this method would prove unsatisfactory. However, it was not known that the amount utilized under the conditions of our tests would be sufficient to exclude this method. In order to study this point the following experiment was conducted:

A series of dilutions from 1:1,000 to 1:4,000,000 of pure indol in physiologic saline was prepared in duplicate. The first set was treated with bacterial sediment. Both sets were incubated for twenty minutes at 37° C. and then tested in the usual manner for indol. In the treated set the highest dilution giving a positive test was 1:500,000, and in the untreated set it was 1:3,000,000. This result indicated sufficient removal of indol as to preclude this direct method of quantitation.

B. Colorimetric comparison between the intensity of reaction in the test solution and that obtained in solutions of pure tryptophan of known strength treated in an identical manner at the same time.

1 Comparison of different sets of solutions of known strength When a series of dilutions of pure tryptophan was prepared in duplicate and both sets treated in the same way at the same time with bacterial sediments of the same batch, not only was the highest dilution giving a positive test identical in the two sets, but the corresponding tubes of each set showed reactions of the same intensity. However, tests carried out on different days or with different batches of bacterial sediment varied considerably. It was obvious, therefore, that no permanent color standard could be devised and that the standards must be prepared at the same time with identical bacterial suspensions and technique.

2 Quantitation of the indol precursor in body fluids and excretions on the basis of tryptophan equivalents. In the manner already described a series of dilutions from 1:1,000 to 1:4,000,000 of pure tryptophan in physiologic saline were prepared side by side with samples of fresh urine and body fluid. All were treated for twenty minutes at 37° C with the same amounts of bacterial sediment from the same batch. Each tube was then tested for indol, and the intensity of color produced in the test samples was compared with that present in the graded series of tryptophan solution.

The results, which are described elsewhere in part<sup>2</sup> showed that the tests in different specimens varied from negative to an intensity greater than that given by the 1:1,000 solution of tryptophan (0.1 per cent).

#### SUMMARY AND DISCUSSION

The results of these experiments have shown that the resting bacteria technique is readily adapted to the study of low concentrations of free tryptophan. Under optimal experimental conditions the conversion of tryptophan into indol was extremely rapid, being detectable almost instantaneously and maximal within twenty minutes. The method was applicable not only to substrates that normally support a vigorous growth of bacteria, but also to many substrates which, for lack of nutritional or electrolytic requirements fail to support such growth.

In general, the results support the findings of Woods<sup>1</sup> and those of Happold and Hoyle,<sup>2</sup> referred to earlier in this report. However, certain notable differences are evident. It will be seen, for example that the speed of the reaction was much greater in the present experiments than in those of the other workers. In attempting to explain this marked difference two possibilities present themselves. The first concerns the indol producing activities of the various cultures employed. While it cannot be said definitely that my strain was more active than those of the other investigators such a possibility can not be excluded, since it is known not only that the strain used in the present experiment was extremely active as compared with other strains but also that this same strain showed considerable variability in its activity from one culture to another.

A second possibility concerns the proportions of bacterial substance to substrate. In the present work optimal results were obtained when the proportion of packed bacterial sediment to total volume of suspension was at least 1 to 16. On this basis it would be necessary to use 75 c.c. of packed sediment in the type experiment of Woods. Whether such a large amount was obtained from 20 Roux bottle cultures cannot be ascertained from the text of his paper.

It should be noted also that the results of one of the present experiments apparently disagrees with Woods' assumption, based on the work of Herzfeld and Klinger,<sup>10</sup> that *Bacterium coli* does not decompose indol, and supports the opposite finding of Logie.<sup>9</sup> While it is impossible from my results to state that indol is actually decomposed, it is certain that appreciable amounts are removed from solution in the heavy suspensions used. It might be asked whether part of the discrepancy between the actual and the theoretical yield of indol in Woods' experiments might not be due to its removal by the organisms rather than entirely to loss from aeration and distillation as suggested.

As pointed out before, this loss of indol was in part responsible for the adoption of a quantitative procedure in terms of tryptophan equivalents rather than the direct quantitation of produced indol in the present work.

### CONCLUSION

The so-called "resting" bacterial suspension technique is adaptable to the identification and rough quantitation of free tryptophan found in urine and body fluids.

### REFERENCES

1. Vaughan, S. L.: The Examination of Urine for Indol, *Proc. Soc. Exper. Biol. & Med.* 29: 623, 1932.
2. Vaughan, S. L.: The Formation of Indol in Urine, *J. LAB. & CLIN. MED.* 22: 390, 1937.
3. Carnes, H. E., and Lewis, G. T.: The Source of Urinary Indol, *J. LAB. & CLIN. MED.* 23: 459, 1938.
4. Woods, D. D.: The Breakdown of Tryptophan by Washed Suspensions of *Bacterium Coli*, *Biochem. J.* 29: 640, 1935.
5. Woods, D. D.: The Action of Washed Suspensions of *Bacterium Coli* on Indol Derivatives, *Biochem. J.* 29: 649, 1935.
6. Happold, F. C., and Hoyle, L.: Enzyme Preparations and Their Action on Tryptophan and Some Indol Derivatives, *Biochem. J.* 29: 1918, 1935.
7. Quastel, J. H., and Whetham, M. D.: The Equilibria Existing Between Succinic, Fumaric and Molic Acids in the Presence of Resting Bacteria, *Biochem. J.* 18: 519, 1924.
8. Vaughan, S. L., and Hubbard, R. S.: Destruction of Reducing Sugars by Resting *Bacterium Coli*, *Proc. Soc. Exper. Biol. & Med.* 30: 405, 1932.
9. Logie, W. J.: On the Synthesis of Tryptophan by Certain Bacteria and on the Nature of Indol Formation, *J. Path. & Bact.* 23: 224, 1930.
10. Herzfeld, E., and Klinger, R.: Quantitative Untersuchungen über den Indol and Tryptophannunsatz der Bakterien, *Centralbl. f. Bakt. (original)* 76: 1, 1915.

# LIPID CHANGES IN NERVE DEGENERATION IN AVITAMINOSIS A\*

## LIPIDS OF SCIATIC NERVE IN AVITAMINOSIS A

RAYMOND REISER A B PH D DURHAM N C

### NERVE DEGENERATION IN AVITAMINOSIS A

SINCE Hart and his co workers<sup>1</sup> noted muscular incoordination in swine on a diet that has since been shown to be deficient in vitamin A several workers have investigated quite thoroughly the histologic changes that occur in the nerves in this condition. Mellanby, by the use of staining methods, demonstrated that the changes take place in the myelin sheath, and concluded that they differ only in degree from typical Wallerian degeneration. This work was confirmed by Zimmerman.<sup>4</sup>

In 1934 Sutton and his co workers<sup>2</sup> published a comprehensive study of nerve degeneration in white rats on vitamin A deficient diets. These authors criticized the staining methods of histologic examination and examined frozen sections in the polarizing microscope. They quoted Cramer and Lee<sup>3</sup> to show that cholesterol and the phospholipids are anisotropic to polarized light and that the triglycerides and fatty acids are isotropic. By this method, they demonstrated the disappearance of anisotropic material in the myelin sheath and verified Mellanby's conclusion that vitamin A degeneration presents a similar picture to Wallerian degeneration in the peripheral nerves. They were able to detect these changes much sooner than by ordinary histologic procedure.

Recently, Helen Gillum and Ruth Okey<sup>7</sup> were able to find no significant variation in the lipid content of brains or any other tissue of white rats on a vitamin A deficient diet.

The nerves used in the present study were obtained from rats used by Dr T S Sutton, who with Dr H E Setterfield, has been continuing his work on nerve degeneration. Dr Sutton has published a complete account of the manner in which these animals were depleted of vitamin A, the grade of standard rat used, and the diet of the controls.<sup>5</sup>

There are, in the literature, three methods for the partition of the lipids in a small amount of nerve material.<sup>8,10†</sup> However, none of these make provision for the determination of triglyceride esters or of bound cholesterol. Furthermore, the last two methods are extremely complicated, and all are open to the criticism that the material is dried before extraction.

\*From the Laboratory of Agricultural Chemistry, Ohio State University.

The data presented in this paper formed part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Ohio State University, 1936.

Received for publication September 2, 1938.

†Since the completion of this study a paper by R. Kirk has appeared in *J Biol Chem* 127: 623, 1938, describing a method of lipid partition which might readily be adapted to nerve material.

In one series of analyses in the present paper, cholesterol was partitioned into free and esterified, and conjugated lipids were separated into lecithin, cephalin, sphingomyelin, and cerebrosides. A large drop was noted in the saturated lipids, and a smaller one in the unsaturated lipids. Cholesterol remained unchanged. None of the changes, however, were significant according to the rule that, to be significant, "a difference in means must be at least one and a half times the sum of the mean deviation from the respective means."<sup>11</sup> Since Dr. Sutton's study with the polarizing microscope has shown that the nerves do not all degenerate to the same degree, the large mean deviation is understandable. Nevertheless, the sum of the fatty acids of sphingomyelin and the cerebrosides showed a difference that would be significant. Also, since it was evident that both fractions of the saturated and unsaturated lipids changed at about the same rate, another series of analyses was made in which the conjugated lipids were partitioned into saturated and unsaturated fractions only. There was a significant drop in the percentage of saturated lipids.

#### METHODS

The sciatic nerves were carefully excised so as to remove all adventitious fat and connective tissue. Upon excision, the material was placed in a small vial previously saturated with water vapor by means of a piece of moistened filter paper. At no time were the nerves kept thus for more than an hour. They were weighed as soon after excision as possible, the weight of the two nerves being usually between 50 and 100 mg.

After weighing, the nerves were transferred to a small mortar and finely ground with five or six times their weight of clean silica sand. The powder was easily transferred to a 50 ml. Erlenmeyer flask with the aid of a small spatula. A little powder remaining was washed into the flask with three 3 ml. portions of 95 per cent alcohol and finally with a little ether.

*Extraction.*—The alcohol-ether mixture was boiled on the steam bath for about a half hour. A 2 inch funnel was put in the flask as an aid in refluxing and as a protection against possible bumping. The solution, after settling of the powder, was carefully decanted into another 50 ml. Erlenmeyer flask. The extraction was repeated twice with 95 per cent alcohol and twice with absolute alcohol. Finally it was extracted twice with chloroform. About 5 ml. of solvent were used each time and boiled for not less than fifteen minutes, or while the previous extract was evaporating to a small volume.

The lipid extract contained, likewise, some pulverized sand and nonlipid material. It was extracted four times with 5 ml. portions of boiling chloroform. The extracts were filtered, in their turn, into a 15 ml. graduated centrifuge tube and boiled to about 0.5 ml. A short piece of melting point tube fused to a thin glass rod was used as an antibumper.

*Precipitation of the Conjugated Lipids.*—Three drops of a saturated solution of strontium chloride in alcohol were added to the chloroform solution of the total lipid, and then 7 ml. of acetone. The mixture was allowed to stand about one hour, after which time the tube was centrifuged. The supernatant solution was transferred to a 50 ml. Erlenmeyer flask, and the conjugated lipids washed twice with 3 ml. volumes of acetone.

*Determination of Free Cholesterol*—The acetone solution and washings from the precipitated phospholipid were evaporated to dryness and the lipid dissolved in 5 ml of petroleum ether. Ten milliliters of a 0.2 per cent solution of digitonin in 50 per cent alcohol were added, and the mixture evaporated to dryness on the steam bath, out of contact with active steam.

After the flask had cooled, bound cholesterol and neutral fat were extracted with several small portions of cold petroleum ether, the extracts being transferred to a 50 ml Erlenmeyer flask. The digitonide need not be disturbed during the extraction. A few flakes that may come loose from the bottom of the flask quickly settle and the ether solution may be transferred by decantation or a dropping pipette without disturbing them.

Ten milliliters of water were added to the digitonide and gently boiled for two minutes. It was allowed to cool, and 20 ml of acetone were added. After thorough mixing, the solution and suspended digitonide were transferred to a 50 ml conical bottom centrifuge tube and the flask washed once with a little acetone. It is not necessary to remove all the digitonide from the flask.

The tube was then centrifuged at about 1500 or 2000 r.p.m. for five or ten minutes and the supernatant solution carefully decanted. A few milliliters of methyl alcohol were boiled in the flask in which the digitonide had been prepared and transferred to the centrifuge tube where they were again boiled. The hot solution was finally transferred to an oxidation flask. The precipitation flask and tube were washed twice with boiling methyl alcohol and the washings added to the oxidation flask. The cholesterol was oxidized according to Boyd.<sup>12</sup>

*Determination of Bound Cholesterol and Neutral Fat Fatty Acids*—The petroleum ether extracts of the free cholesterol digitonide contained bound cholesterol and neutral fat. The petroleum ether was evaporated and the lipid dissolved in 5 ml of alcohol and saponified on the steam bath with 0.1 ml of 50 per cent potassium hydroxide for one hour. When the alcohol had evaporated until no odor remained, the soaps were acidified with 1 ml of 20 per cent phosphoric acid. The acid solution was heated on the steam bath for a few minutes and the lipid extracted several times with boiling petroleum ether. The ether extracts were transferred to a 50 ml Erlenmeyer flask and 2 or 3 ml of digitonin solution were added. The flask was placed on the steam bath until dry. The fatty acids were extracted from the digitonide in the same manner as had been the fatty acid esters from the free cholesterol digitonide and transferred to an oxidation flask. The bound cholesterol digitonide was treated exactly as the free.

*Determination of Conjugated Lipids*—In the first series of analyses the conjugated lipids were fractionated into lecithin, cephalin, sphingomyelin, and cerebrosides in the following manner:

The acetone insoluble material, which contained the cerebrosides and the strontium chloride salts of the phospholipids, was dissolved in 0.2 ml of chloroform, and 0.2 ml of ammoniated methyl alcohol was added to decompose the salt. The ammoniated methyl alcohol was prepared by dissolving 15 ml of 29 per cent ammonium hydroxide in 86 ml of methyl alcohol. This makes, approximately, a 5 per cent solution of ammonia.



The ammoniated solution was allowed to remain for a short time at room temperature. The chloroform, alcohol, and ammonia were then drawn off with reduced pressure. Bumping was prevented by holding the tube partially on its side and rotating it.

The dried mixture was then dissolved in a minimum volume of chloroform (not more than 0.2 ml.), and about 5 ml. of petroleum ether were added. Sphingomyelin and cerebrosides precipitated, while the unsaturated lipids, lecithin, and cephalin, remained in the solution. The tubes were centrifuged at about 2,000 r.p.m. until the supernatant solution was clear. The petroleum ether solution was transferred into another centrifuge tube, and the centrifugate washed with 3 ml. of petroleum ether. The petroleum ether solution of the lecithin and cephalin was evaporated on the steam bath while the suspended sphingomyelin and cerebrosides were being centrifuged from the petroleum ether washings. This washing was then added to the lecithin and cephalin and boiled to about 0.2 ml.

Five milliliters of absolute alcohol were added to this petroleum ether solution and the cephalin precipitated. After centrifuging, the supernatant solution was decanted into a 50 ml. Erlenmeyer flask. The cephalin was washed once with 3 ml. of absolute alcohol and the washings (separated by centrifuging) added to the lecithin solution. The cephalin was transferred to another 50 ml. Erlenmeyer flask by means of chloroform.

The sphingomyelin-cerebroside mixture was then extracted twice with 3 ml. portions of cold pyridine. This solvent dissolves the cerebrosides but not the sphingomyelin.<sup>13</sup> The washings were transferred to a 50 ml. Erlenmeyer flask. The sphingomyelin was dissolved in chloroform and also transferred to a 50 ml. Erlenmeyer flask.

The solutions were evaporated to dryness and saponified in 95 per cent alcohol, with 0.1 ml. of saturated potassium hydroxide. The extracted fatty acids were determined by oxidation.

In the second series of analyses, the petroleum ether soluble and insoluble fractions were not subdivided, but each was transferred to a 50 ml. Erlenmeyer flask and saponified. The fatty acids were made up to volume with petroleum ether in a 25 ml. volumetric flask, and an aliquot removed for oxidation.

*Calculations.*—The fatty acids of lecithin and cephalin were calculated as being oleic, and those of sphingomyelin and the cerebrosides as lignoceric. To calculate the weight of each of the conjugated lipids, the weight of its constituent acid was multiplied by the following factors:

Lecithin	1.39	Sphingomyelin	2.14
Cephalin	1.32	Kerasin	2.16

In the last set of analyses, when the lipids were divided into only the saturated and unsaturated fractions, the factor 2.15 was used for the former and 1.39 for the latter.

Neutral fat was calculated by multiplying the number of grams of neutral fat fatty acids, figured as oleic, by the factor 1.04.

Free and bound cholesterol were calculated by multiplying the number of milliliters of 0.1 N potassium permanganate required to oxidize the digitonide by the factor 0.0976

Results—Tables I, II, and III show the results of analyses of normal and degenerated nerves

TABLE I

NERVE ANALYSES IN WHICH THE CONJUGATED LIPIDS WERE FRACTIONATED\*

RAT	NEUTRAL FAT	FREE CHOLESTEROL	BOUND CHOLESTEROL	LECITHIN	CEPHALIN	SPHINGO MYELIN	KEPASIN
Normal	0.70	3.12	0.159	6.34	2.86	1.62	5.93
Normal	1.53	2.92	0.137	6.47	2.80	1.32	5.60
Normal	0.98	2.41	0.148	—	—	—	—
Normal	1.06	3.50	0.190	6.55	4.98	1.62	4.54
Normal	2.48	3.47	—	8.04	3.36	1.70	5.52
Mean	1.35	3.08	0.15	6.85	3.50	1.56	5.42
<i>First Group, Degeneration of Avitaminosis A</i>							
1116	0.81	3.50	0.119	5.94	4.76	0.38	3.08
1117	—	3.89	—	—	4.44	1.09	3.17
1084	0.30	2.59	0.121	5.60	5.99	1.85	5.55
1085	0.41	2.63	0.074	4.81	3.56	0.77	3.26
1093	1.86	3.16	0.180	—	4.14	1.68	2.79
1103	2.01	3.20	0.306	6.99	2.29	1.99	2.06
Mean	1.08	3.42	0.160	5.85	4.18	1.29	3.32
Per cent change	-20.0	+11.0	+4.57	14.6	+19.4	17.3	38.7
<i>Second Group, Degeneration of Avitaminosis A</i>							
E4477	2.62	2.53	0.331	6.45	3.45	1.93	—
E4452	2.94	2.45	—	6.04	4.61	1.09	2.32
E4455	3.45	2.41	0.104	5.06	2.05	1.44	3.15
E4476	1.71	2.36	0.048	5.61	1.23	0.54	3.32
E4481	2.60	2.44	0.171	7.83	3.12	0.86	4.84
Mean	2.66	2.44	0.167	6.20	2.90	1.17	3.41
Per cent change	+97.0	-20.8	+6.54	9.49	17.1	-25.0	-37.0

\*Figures represent per cent of moist weight

TABLE II

NERVE ANALYSES IN WHICH CONJUGATED LIPIDS WERE FRACTIONATED INTO SATURATED AND UNSATURATED GROUPS ONLY

RAT	NEUTRAL FAT	BOUND CHOLESTEROL	FREE CHOLESTEROL	SATURATED CONJUGATED LIPID	UNSATURATED CONJUGATED LIPID
Normal	0.60	—	—	4.60	11.4
Normal	0.50	—	—	5.80	10.6
Normal	—	—	—	5.07	11.1
Normal	—	—	—	5.20	11.1
Normal	0.79	0.18	3.73	6.17	10.5
Normal	1.69	1.16	3.56	6.02	—
Normal	2.18	0.21	2.86	5.40	12.2
Normal	1.67	0.20	2.73	5.68	10.6
Mean	1.22	0.19	3.22	5.49	11.1
<i>Degeneration of Avitaminosis A</i>					
1121	1.19	0.30	2.55	4.45	7.84
1127	0.99	0.24	3.30	4.19	12.40
1124	0.69	—	2.48	3.93	7.84
1125	0.98	0.32	—	3.57	13.40
1129	0.48	0.31	2.73	4.44	11.00
Mean	0.87	0.29	2.77	4.15	10.34
Per cent change	+28.7	+0.53	-14.0	-24.4	-7.20

TABLE III

A COMPARISON OF THE NORMAL NERVE MATTER GIVEN IN THE LITERATURE WITH THAT DETERMINED BY THE AUTHOR

INVESTIGATOR	CHOLESTEROL		PHOSPHOLIPID			CEREBROSIDE	TOTAL LIPID
Smith and Mair <sup>23</sup>	4.6		7.6			4.1	19.7
			5.65				18.8
			7.6				20.5
Koch and Koch <sup>24</sup>	2.00		5.39			3.24	19.5
	1.98		6.00				17.2
Koch and Voeglin <sup>22</sup>	3.34		8.52			3.36	18.4*
Jordan, Randall, and Bloor <sup>14</sup>	1.36		4.40			1.73	15.8†
Mott and Halliburton <sup>16</sup>			9.49				
			Leci- thin	Cepha- lin	Sphingo- myelin		
Koch <sup>25</sup>	4.86		5.19	3.49		4.57	19.5
	Free	Bound					
Verzár, Arvay, and Kokas <sup>26</sup>	4.08	0.66					
Gassner <sup>27</sup>	1.25	0.15					
		0.31					
		0.1					
Reiser	3.08	0.15	6.85	3.50	1.56	5.32	21.9‡
	3.22	0.19	11.1		5.49		

\*Includes 3.49 per cent of sulfatides.

†Includes 8.35 per cent triglycerides.

‡Includes 1.21 per cent triglycerides.

## DISCUSSION

The results of the analyses of normal nerves agree closely with the figures in the literature and are compared with those figures in Table III. An exception to this close agreement is the analyses of Jordan, Randall, and Bloor.<sup>14</sup> These authors obtained values higher in triglycerides and lower in all other lipids. Perhaps this is because they used methods intended for other kinds of tissues which contain very different proportions of the lipids. The other analyses reported were made by the use of methods especially designed for nerve material.

The evidence of the analyses presented above does not support the reasoning of Setterfield and Sutton<sup>15</sup> that the conjugated lipids are replaced by neutral fat in nerve degeneration due to vitamin A deficiency. There appears to be a significant increase in neutral fat in the E group of animals in the first series of analyses. However, it must be borne in mind that this fraction may not be nerve material at all but fat from connective tissue which may have adhered to the nerve during its excision. The animals of the E group were unusually fat for vitamin A deficient animals and one or two per cent more fat may have adhered to these nerves. It is also possible that this fraction was not neutral fat but unsaturated cerebrosides soluble in acetone.

The changes in free and esterified cholesterol cannot be considered as significant. Even though in the second series the cholesterol ester increased 50 per cent and the per cent deviation was very small, the total amount is so low that a larger series of determinations would have to be made before any significance could be attached to the increase.

The changes in the conjugated lipids present an interesting study. In all analyses, the petroleum ether insoluble lipids, which are considered to be sphingomyelin and kersin, decreased considerably. Yet the petroleum ether soluble

phosphatides showed no significant decrease, but did, in one series, increase as much as 19 per cent

The decrease of phosphatides and then replacement with neutral fat can not, therefore, account for the disappearance of the anisotropic material in these degenerating nerves. This conclusion can be supported by evidence from the literature. Thus, Mott and Halliburton<sup>16</sup> and May<sup>17</sup> were able to find no decrease in the lipid phosphorus of the sciatic nerve during the first seven days of Wallerian degeneration. Yet Setterfield and Sutton<sup>18</sup> have shown that about half of the anisotropic material has disappeared during this time. Mott and Halliburton also noticed a positive Marchi reaction in degenerating nerves before there was any change in phosphorus content.

It is possible that the paradoxes can be explained on the basis that in a normal functioning nerve, the conjugated lipid material is in an organized form, perhaps in some conjugation with protein. It is this organized material that is birefringent and fails to stain with the fat stains. The first stage of nerve degeneration may then be postulated as being a breakdown of this organization. The lipids are still in the nerve, but are nonfunctional, do not rotate polarized light, and can be stained. The fact that the organized fats of living tissues do not stain is well known and has been emphasized by Wells.<sup>19</sup>

The next step is the removal of the lipids from the nerve. In accord with the evidence presented in this paper, the stable saturated lipids may be converted to the more labile unsaturated ones and thus be removed.

Evidence that phospholipid is increased in degenerating nerve material has been presented in the literature. Pighini and Fontesesi<sup>19</sup> have shown that phospholipids increase during the aseptic autolysis of the spinal cord of a dog. Backlin<sup>9</sup> found a decrease in cerebrosides and a possible increase in the phospholipids of brain during autolysis, and Singer<sup>20</sup> found an increase in phospholipid content of the frontal lobes in progressive paralysis. Koeh,<sup>21</sup> however, found a decrease in phospholipid in the central nervous system during general paralysis. Koeh and Voeglin<sup>22</sup> observed a much greater decrease in the cerebrosides than in the phospholipids in the spinal cord in pellagra.

#### SUMMARY AND CONCLUSIONS

- 1 A new micromethod for the partition of the lipids in nerve material has been developed.
- 2 An acetone soluble material, not cholesterol, was found in the sciatic nerves of albino rats.
- 3 There is a decrease in the saturated conjugated lipids, calculated as sphingomyelin and kersin, during nerve degeneration due to lack of vitamin A.
- 4 There is no decrease in lecithin and cephalin during nerve degeneration due to lack of vitamin A.
- 5 Bound cholesterol was found in nerve tissues.
- 6 Free cholesterol does not change significantly during nerve degeneration due to lack of vitamin A.
- 7 The significance of these findings was discussed.

The author is deeply obligated to Professor J. F. Lyman who suggested this work and whose able counsel was always available. He also appreciates the assistance of Dr. T. S. Sutton who supplied the nerve material.

## REFERENCES

1. Hart, E. B., Miller, W. B., and McCollum, E. V.: Further Studies on the Nutritive Deficiencies of Wheat and Grain Mixtures and the Pathological Conditions Produced in Swine by Their Use, *J. Biol. Chem.* 25: 239, 1916.
2. Mellanby, E.: Xerophthalmia, Trigeminal Degeneration and Vitamin A Deficiency, *J. Path. & Bact.* 38: 391, 1934.
3. Mellanby, E.: Lesions of the Central and Peripheral Nervous System Produced in Young Rabbits by Vitamin A Deficiency and a High Cereal Intake, *Brain* 58: 141, 1936.
4. Zimmerman, H. M.: Lesions of the Nervous System in Vitamin Deficiency, *J. Exper. Med.* 57: 215, 1933.
5. Sutton, T. S., Setterfield, H. E., and Kraus, W. E.: Nerve Degeneration Associated With Avitaminosis in the White Rat, *Ohio Agri. Exper. Sta., Bull.* No. 545, 1934.
6. Lee, A. B.: *Microtommists Vade Mecum*, Philadelphia, 1921, P. Blakiston's Sons & Co.
7. Gillum, Helen, and Okey, Ruth: Effect of Quantitative Underfeeding and of Vitamin A Deficiency on the Tissue Lipids of Rats Fed Diets Low in Cholesterol, *J. Nutrition* 11: 303, 1936.
8. Gorodissky, H.: Zur Micromethodik der quantitativen Bestimmung der Hirnlipide, *Biochem. Ztschr.* 159: 379, 1925.
9. Backlin, E.: Beitrage zur quantitativen Kenntnis der Gehirnlipide, *Upsala Läkaref. förh.* 35: 105, 1930.
10. Mitolo, M.: Technic for the Microdetermination of the Various Lipoidal Fractions in the Central Nervous System, *Boll. soc. ital. biol. sper.* 8: 69, 1933; *Chem. Abst.* 27: 3963, 1933.
11. Scott, E. L.: What Constitutes an Adequate Series of Physiological Observations? *J. Biol. Chem.* 73: 81, 1931.
12. Boyd, E. M.: Low Phosphorus Values in Dog Plasm, *J. Biol. Chem.* 91: 1, 1931.
13. Rosenheim, O.: The Galactosides of the Brain, *Biochem. J.* 7: 604, 1913.
14. Jordan, W. R., Randall, L. O., and Bloor, W. R.: Neuropathy in Diabetes Mellitus, Lipid Constituents of the Nerves Correlated With the Clinical Data, *Arch. Int. Med.* 55: 26, 1935.
15. Setterfield, H. E., and Sutton, T. S.: The Use of Polarized Light in the Study of Myelin Degeneration. I. The Appearance and Progress of Degeneration After Transection of the Sciatic Nerve of the White Rat, *Anat. Rec.* 61: 397, 1935.
16. Mott, F. W., and Halliburton, W. D.: The Physiological Action of Choline and Neurine, *Tr. Roy. Soc. London*, s. B. 191: 211, 1899.
17. May, R. M.: Etudes microchimiques sur le système nerveux. III. L'eau et les combinaisons phosphorées du nerf au cours de sa dégénérescence, *Bull. Soc. chim. biol.* 12: 934, 1939.
18. Wells, H. B.: *Chemical Pathology*, Philadelphia, 1925, W. B. Saunders Co.
19. Pighini, G., and Fontesesi, C.: Über die Autolyse des Nervengebes, *Biochem. Ztschr.* 63: 336, 1914.
20. Singer, K.: Über die Phosphatide der Petrolätherfraktion des Gehirns bei progressiver Paralyse und bei Marasmus, *Biochem. Ztschr.* 198: 340, 1928.
21. Koch, M. L.: Chemical Investigations of the Central Nervous System in Two Cases of General Paralysis, *Arch. Neurol. & Psychiat.* 7: 488, 1922.
22. Koch, M. L., and Voeglin, C.: Chemical Changes in the Central Nervous System as a Result of Restricted Vegetable Diet, *U. S. Hygienic Lab. Bull.* No. 103, 1916.
23. Smith, J. L., and Mair, W.: On a Method of Quantitative Analysis of Tissue Lipids, With a Preliminary Note on Degeneration of the Brain, *J. Path. & Bact.* 16: 131, 1911.
24. Koch, W., and Koch, M. L.: Contributions to the Chemical Differentiation of the Central Nervous System. IV. The Relative Amount of Sheathing Substance in the Corpus Callosum and the Intradermal Nerve Roots (Man and Dog), *J. Biol. Chem.* 31: 395, 1917.
25. Koch, W.: Methods for the Quantitative Chemical Analysis of the Brain and Cord, *Am. J. Physiol.* 11: 303, 1904.
26. Verzár, F., Kokas, E., and Árvay, A.: Die Bindung des Cholesterins im Nervensystem bei Mangel an Vitamin B, *Pflüger's Arch. f. d. ges. Physiol.* 206: 666, 1924.
27. Gassner, L.: Der Gehalt des Nervensystems an gebundenem Cholesterin, *Biochem. Ztschr.* 180: 359, 1937.

# METASTATIC BONE CARCINOMATOSIS ASSOCIATED WITH PARATHYROID HYPERPLASIA AND WITH SYMPTOMS OF HYPERPARATHYROIDISM\*

WITH BLOOD CALCIUM AND PHOSPHORUS STUDIES BEFORE AND AFTER EXTIRPATION OF A HYPERPLASTIC PARATHYROID GLAND

S BEN ASHFR, M D, JERSEY CITY N J

THE role of the parathyroid glands in calcium metabolism is now well recognized. The investigations of Hanson<sup>1</sup> and Collip<sup>2</sup> have demonstrated that these glands produce a hormone whose function is to regulate the calcium level of the blood. Injection of this hormone in animals and in the human being produces a rise in the level of the serum calcium.<sup>3</sup> Extirpation of the parathyroid glands produces a state of tetany with a fall of serum calcium. In certain pathologic states alterations of the parathyroid glands sometimes occur. In osteitis fibrosa cystica, or hyperparathyroidism, in which condition there is a constant hypercalcemia, an adenoma of a parathyroid gland is usually found. This adenoma is considered to be the primary and etiologic factor in the production of the disease. Removal of the adenoma is followed by clinical improvement, with a fall of the blood calcium to normal levels. The disease has been produced experimentally by the prolonged administration of parathyroid hormone.<sup>3</sup>

The syndrome of hyperparathyroidism is now a well recognized clinical entity. The diagnosis is based upon a history of renal calculi and arthritic pains, the presence of hypercalcemia, hypophosphatemia, and an increased excretion of calcium in the urine, the finding of muscular hypotonia, widespread rarefaction of the bones, and a parathyroid tumor. Of these the hypercalcemia and the changes in the bones are the most characteristic findings. The presence of a high serum calcium in a patient with muscular hypotonia, or arthritic pains, should lead one to suspect hyperparathyroidism.

Although the x-ray of the bones and the blood findings are characteristic, confirmation of the diagnosis is sometimes very difficult, as evidenced by reports in the literature of cases operated upon several times in an attempt to find the adenoma. The difficulties encountered in the differential diagnosis are exemplified by a case reported by Mason and Warren.<sup>4</sup> Their patient presented widespread bony changes, hypercalcemia, and a negative calcium balance. Al-

\*From the Medical Departments of the Greenville Hospital and the Medical Center Jersey City.

Received for publication September 20 1933.

†The rise is either the result of a direct action of the hormone on the calcium or as Albright<sup>5</sup> suggests the hormone first causes a fall in the blood phosphorus followed by a secondary rise in the calcium.

though the x-ray examination of the bones suggested metastatic carcinoma, a search was made for a parathyroid adenoma. At the operation a metastatic carcinoma to the thyroid and parathyroid glands was found which originated from a mammary cancer removed five years before. The hypercalcemia, they state, was either due to an associated hyperparathyroidism, or was an accompaniment of widespread bone metastasis.

In rickets, osteomalacia, and osteogenesis imperfecta, in which conditions there is a low level of serum calcium, hyperplasia of the parathyroid is often found. This hyperplasia is considered to be secondary in nature.

In 1923 Klemperer<sup>5</sup> reported a case of breast carcinoma with metastases to the bones associated with parathyroid hyperplasia. Barr and Bulger<sup>6</sup> in 1930 reported a case of multiple myeloma with parathyroid hyperplasia, hypercalcemia, a negative calcium balance, increased urinary calcium and metastatic calcification in the lungs, stomach, and kidneys. They considered the hyperplasia as secondary to the bone changes, and the hypercalcemia as a manifestation of increased function of the parathyroid glands.

Thus we find two instances in the literature of parathyroid hyperplasia occurring secondary to widespread bone destruction: one case, that of multiple myeloma, was associated with hypercalcemia and other symptoms of hyperparathyroidism; in the other case of metastatic carcinoma there were no calcium studies recorded.

A case is presented here with definite bone metastases associated with parathyroid hyperplasia, and with symptoms of hyperparathyroidism. Blood calcium studies were made before and after the extirpation of a hyperplastic parathyroid gland.

#### CASE REPORT

*Clinical History:* Patient A. G., a man aged 58 years, was admitted to the Greenville Hospital on Nov. 5, 1935, complaining of pain in the right shoulder and right arm, right hip, and right leg. The family history was insignificant. His previous history was negative, except for "flu" in 1918. There was no history of any venereal infection. The patient was in good health until about two months previously, when he began to have pain in the right shoulder and right arm. He consulted a physician who informed him that he had "neuritis." About one month later he began to have pain in the right hip radiating down the right leg. His pain gradually became more severe, and he lost about 10 pounds in weight. There were no gastrointestinal, respiratory, nor genitourinary symptoms.

Physical examination revealed a rather thin, somewhat emaciated adult. The muscles were rather flabby. There was slight limitation of motion about the right shoulder joint. The thyroid gland was not enlarged. There were no masses nor enlarged glands felt in the neck. The heart and lungs were normal. Examination of the abdomen did not reveal anything abnormal. The prostate gland was not enlarged. There were no nodules in the axillae nor in the supraclavicular regions. The reflexes were normal. The blood pressure was 120/80.

The laboratory examination revealed the following: The urine was negative for sugar, albumin, and Bence-Jones protein. There were no red and white blood cells. An occasional hyaline cast was seen. The blood count showed a hemoglobin of 80 per cent, red blood cells 4,580,000, white blood cells 8,800, polymorphonuclear cells 70 per cent, small lymphocytes 21 per cent, monocytes 6 per cent, and eosinophiles 3 per cent. The blood chemistry showed nonprotein nitrogen 32 mg., urea nitrogen 16 mg., calcium 14.2 mg., phosphorus 2.4 mg. per 100 c.c. of blood. The phenolphthalein excretion was 60 per cent in two hours. The Wassermann and Kahn tests were negative.

X ray studies of the genitourinary tract, chest, and gastrointestinal tract gave negative results. X ray examination of the right humerus (Fig 1) showed a large multilocular cyst in the upper third of the shaft with expansion and thinning of the cortex. There was some rarefaction of the bone below the cyst. X ray of the skull (Fig 2) showed multiple areas of rarefaction. These areas varied in size from  $\frac{1}{8}$  inch to 1 inch in diameter. The surrounding structure showed no changes. There was some rarefaction in the bones of the pelvis and right femur and in both fibulae. These findings suggested bone metastases, although rarefaction due to hyperparathyroidism could not be excluded.

On November 9 the patient was placed on a low calcium diet and after three days the calcium and phosphorus in the urine and stool were determined for the following three days. The results of these studies are shown in Tables I, II, and III.

TABLE I  
CALCIUM AND PHOSPHORUS INTAKE

DATE	CALCIUM	PHOSPHORUS
	mg	mg
November 9	106	144
November 10	110	410
November 11	120	150
November 12	110	112
November 13	118	360
November 14	102	126

TABLE II  
CALCIUM INTAKE AND OUTPUT

DATE	CALCIUM INTAKE	CALCIUM OUTPUT	
		URINE	STOOL
	mg	mg	mg
November 12	110	106	224
November 13	118	124	366
November 14	102	141	266
	330	376	956
Total calcium intake		300 mg	
Total calcium output		471 mg	
Negative balance		141 mg	

TABLE III  
SERUM CALCIUM AND PHOSPHORUS

DATE	SERUM CALCIUM	SERUM PHOSPHORUS
	mg	mg
Nov 6, 1935	14.2	2.4
Nov 8, 1935	13.8	2.6
Nov 10, 1935	14.0	2.2
Nov 12, 1935	14.6	2.0
Nov 13, 1935	16.2	1.1
Nov 14, 1935	14.2	2.0
Nov 15, 1935	14.9	1.8
Operation Nov 18, 1935		
Nov 22, 1935	14.0	2.4
Nov 27, 1935	13.2	2.0
Dec 16, 1935	12.6	3.2
Dec 23, 1935	11.4	2.4
Jan 8, 1936	12.6	3.2
Jan 27, 1936	13.2	2.2
Feb 4, 1936	13.0	2.6
May 6, 1936	13.4	2.8



It was evident from these studies that the patient had a persistent hypercalcemia. The blood phosphorus was low. The normal relationship of a higher calcium excretion in the feces was reversed, so that there was an increased ratio of calcium in the urine to the total amount of calcium excreted. The patient was also in a negative calcium balance. Because of these findings, an exploration for a parathyroid tumor was deemed warranted.

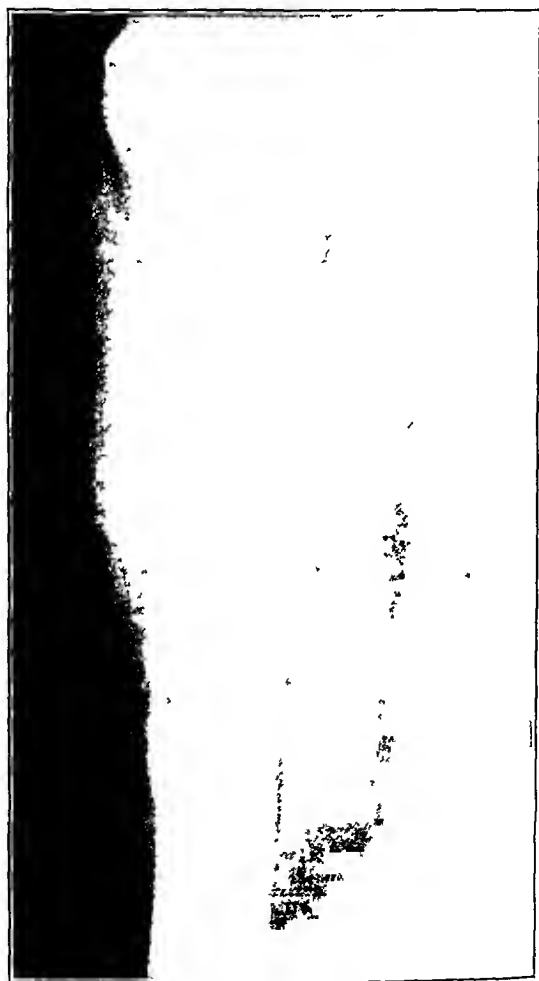


FIG. 1—Roentgen ray of right humerus, showing multilocular cyst formation

Operation was performed by Dr. H. Jaffe on Nov. 18, 1935. The following is the report: "Under general anaesthesia of gas and ether, a typical collar incision was made as for thyroid. In the region of the right upper pole somewhat posteriorly a rounded nodule, the size of a hazel nut, was felt. The right lobe was grasped with a Lahey goiter forceps and the lobe completely dislocated, thus allowing access to the posterior surface. In the region of the inferior pole what appeared to be an enlarged parathyroid gland was found. This was excised as well as the nodule within the upper pole of the lobe. The small mass of tissue

measured 9 by 4 by 5 mm and the larger 18 cm in diameter. The same procedure was followed on the left side, but no masses were found. The glands were replaced and a careful search below the region of the isthmus and in the upper thoracic space was made. No other abnormally situated enlarged parathyroid was found."



Fig 2—Lateral view of skull showing areas of rarefaction

The following is the pathologic report of the excised specimens (Dr W Antopol)

*Gross Examination* (a) A small nodule of tissue, measuring 7 by 2 by 4 mm,\* appears to be encapsulated. On cut section this shows a brownish homogeneous color. (b) A mass of tissue, about 1.5 cm in diameter, which on cut sections shows extensive colloid areas as well as small areas of hemorrhage.

*Microscopic Examination* (Fig 3) (a) Reveals the smaller nodule to be composed of polyhedral cells with abundant clear cytoplasm and a single round nucleus which contains considerable chromatin. These cells are arranged in the form of nests and alveoli. No glandular or cystic elements are noted. (b) Reveals thyroid tissue with a considerable amount of colloid in distended alveoli, areas of hemorrhage, and fibrosis.

*Diagnosis* Hyperplastic parathyroid. The patient stood the operation very well. The next day he was quite comfortable. For the remaining days in the hospital he complained of pain in the right shoulder and right leg. His blood calcium was still high. He refused to stay in the hospital any longer and was discharged on Nov. 27, 1935.

Examination of the patient on Dec. 16, 1935, at the office showed that he was gradually getting worse. The pain in the right shoulder and right leg was very severe. His appetite was fair, but he felt weaker. His blood calcium was 12.4 mg and his blood phosphorus was 2.6 mg.

It was felt that a re-exploration might reveal another parathyroid enlargement which when removed would result in improvement. On Jan. 8, 1936, he was admitted to the Medical Center. An operation was performed by Dr R. Lohman on Jan. 10, 1936. The neck and upper thoracic space were thoroughly re-explored, but no other parathyroid enlargement was discovered.

\*The discrepancy in the measurements is due to the shrinkage of the tissue in the formalin.



Fig. 3.—Microscopic appearance of the hyperplastic parathyroid gland.



Fig. 4.—Microscopic appearance of a biopsy of the right humerus, showing metastatic carcinoma

A biopsy of the right humerus was taken and following is the pathologic report. (Dr. N. Alter):

Microscopic section of the bone marrow (Fig. 4) shows no characteristic tissue elements. These have been replaced by a neoplastic growth which consists of large, pale, somewhat elongated, epithelial cells. There is great variety in shape and size of cells and nuclei. The epithelial cells form solid cords and masses. There is no evidence of keratinization, but there are areas of necrosis where polynuclears are present; otherwise, there is little fibrous stroma with lymphocytic reaction.

*Microscopical Diagnosis* Metastatic carcinoma of bone marrow

The patient was discharged on Jan 19, 1936. Examination of the patient on May 6, 1936, at the office showed that the patient had lost a great deal of weight and felt very weak. He complained of a cough which began three weeks before. There was moderate dullness in the right infraclavicular region with bronchovesicular breathing. The pulse was weak and rapid.

An x-ray of the chest revealed an irregular dense shadow, 2 1/2 inches in length and 1 inch in width, beginning at the right hilus and extending upward toward the right clavicle. Its margin was irregular due to small spurs spreading out into the adjacent tissue. The appearance of the shadow was that of an expanding lesion.

Examination of the sputum was negative for tubercle bacilli. He refused hospitalization and bronchoscopy examination. It was believed that the lesion was a bronchiogenic carcinoma, with metastases to the bones.

On May 21, 1936, the patient had a profuse hemoptysis. The signs in the chest were more marked. He was confined to bed, with repeated attacks of hemoptysis. He gradually became weaker, and died on Nov 5, 1936, at his home. Permission for autopsy was not granted.

## DISCUSSION

The case presents many points of interest. It has generally been accepted that hypercalcemia associated with rarefaction of the bones is pathognomonic of hyperparathyroidism. The parathyroid hyperplasia or adenoma found in this condition is the primary cause, and the clinical symptoms are the result of increased parathyroid function. In the case presented here the hypercalcemia was found to be associated with metastatic carcinoma of the bones and with parathyroid hyperplasia. A search through the literature fails to reveal a similar case with blood calcium studies following the removal of a hyperplastic parathyroid.

The question arises whether the hypercalcemia was the result of hyperfunction of the parathyroid, or whether it was due to excessive bone destruction. Barr and Bulger believe that in their case of multiple myeloma, the hypercalcemia may have reflected increased function of the parathyroid glands.

It will be noted that in our case the removal of the hyperplastic parathyroid gland did not reduce the serum calcium to normal, but the calcium level was somewhat lower than before the operation. It should also be noted that the lowering of the serum calcium was observed ten days after operation and lasted for about ten weeks, after which there was a slight rise. It seems reasonable to assume that both the bone destruction and the hyperplasia may have been factors in producing the hypercalcemia, and, as Barr and Bulger suggest, the bone changes may have stimulated the parathyroid which in turn aggravated the condition. It must be conceded, however, that the failure to find another pathologic parathyroid in the second attempt does not eliminate the possibility of an abnormally situated one, which would account for the persistence of the hypercalcemia.

It would further be of interest to discuss the nature of the parathyroid hyperplasia. The finding of a parathyroid tumor in a case of osteitis fibrosa cystica was first reported by Askanazy<sup>7</sup> in 1904. Erdheim<sup>8</sup> later noted the frequent occurrence of parathyroid hypertrophy in rickets, and concluded that the hypertrophy represented an attempt at compensation for the calcium changes in the blood. This theory was generally accepted and applied also to osteitis fibrosa

cystica. Hoffheinz<sup>9</sup> later pointed out that in practically all cases showing parathyroid hyperplasia, only one gland was found involved, which fact is a strong argument against the theory of compensation.

In 1925 Mandl<sup>10</sup> transplanted four normal human parathyroid glands from a patient dying as a result of an accident into the abdominal wall of a young man with osteitis fibrosa cystica. The patient instead of getting better became worse. A month later Mandl explored the neck and found one of the parathyroid glands to be the site of an adenoma. The gland was removed, followed by striking symptomatic improvement. He thus showed that the changes in the parathyroid gland in osteitis fibrosa cystica were the cause rather than the result of the alteration in the bones.

Klemperer suggests that in his case of bone carcinomatosis, the hyperplasia might be considered as a fruitless attempt of the body to compensate for the loss of calcium, and that it might be conceived as a hypofunctioning rather than a hyperfunctioning gland.

Barr and Bulger believe that in their case of multiple myeloma, the hyperplasia of the parathyroids is secondary to the bone changes and that the hypercalcemia is a manifestation of hyperfunction of the glands.

It is obvious that in our case, also, the hyperplasia was secondary to the bone changes. Since the removal of the gland resulted in somewhat lower level of the blood calcium, it is reasonable to conclude that the gland showed evidence of hyperfunction. This would support the theory of Erdheim of compensatory hyperplasia.

We thus find compensatory hyperplasia in conditions associated with low serum calcium, such as rickets and osteomalacia, and also in states where the serum calcium is high. This apparent paradox is difficult to explain. Further studies of parathyroid physiology might throw more light on this subject.

#### SUMMARY

A case is presented with metastatic carcinoma associated with parathyroid hyperplasia and with symptoms of hyperparathyroidism, such as hypercalcemia, a negative calcium balance, and increased excretion of calcium in the urine. Removal of the gland resulted in a somewhat lower but not normal level of the serum calcium. The nature of hypercalcemia and the hyperplasia is discussed.

I wish to thank Dr. H. Jaffe, Dr. R. Lobban, Dr. Wm. Antopol, and Dr. N. Alter for their invaluable aid in this case.

#### REFERENCES

1. Hanson, A. M.: The Hydrochloric Sicca; a Parathyroid Preparation for Intramuscular Injection, *Mil. Surgeon* 54: 218, 1924.  
Parathyroid Preparations, *Mil. Surgeon* 54: 554, 1924.  
Experiments With Active Preparations of Parathyroid Other Than That of Desiccated Glands, *Mil. Surgeon* 55: 701, 1924.
2. Collip, J. B.: Extraction of a Parathyroid Hormone Which Will Prevent or Control Parathyroid Tetany and Which Regulates the Level of Blood Calcium, *J. Biol. Chem.* 63: 395, 1925.
3. Jaffe, H. L., Bodansky, A., and Blair, J. E.: The Sites of Decalcification and of Bone Lesions in Experimental Hyperparathyroidism, *Arch. Path.* 12: 715, 1931.
4. Mason, R. L., and Warren, S.: Metastatic Carcinoma Simulating Hyperparathyroidism, *Am. J. Path.* 7: 415, 1931.
5. Klemperer, P.: Parathyroid Hyperplasia and Bone Destruction in Generalized Carcinomatosis, *Surg., Gynec. & Obst.* 36: 11, 1923.

- 6 Barr, D P, and Bulger, H A The Chancal Syndrome of Hyperparathyroidism, *Am J M Sc* 179 449, 1930
- 7 Askanazy, M Ueber Ostitis deformans ohne osteoides Genebe *Arch u d Geb d path Anat Inst z Tubingen, Leipzig* 4 399 1904
- 8 Erdheim J Denkschr d k Akad der Wissensch, Math naturw Klasse, Wien, 1914, p 90
- 9 Hoffmeiz Ueber Vergrosserungen der Epithellorperchen bei Ostitis fibrosa und verwandten Krankheitsbildern, *Virchows Arch f path Anat* 256 705, 1925
- 10 Mandl, F Therapeutischer Versuch bei einem Falle von ostitis fibrosa generalisata mittels Extirpation eines Epithellorperchentumors, *Zentralbl f Chir* 53 260, 1926
- Klinisches und Experimentelles zur Frage der lokalisierten und generalisierten Ostitis fibrosa *Arch f klin Chir* 143 1, 1926
- 11 Albright, F, Bruer, W, Ropes, M, and Aub J C *J Clin Investigation* 7 139, 1929
- Albright, F, and Ellsworth R *J Clin Investigation* 7 199 1929
- 12 Cantorow, A Calcium Metabolism and Calcium Therapy Philadelphia 1933, Lea & Febiger

## THE REVERSAL OF KIDNEY VOLUME RESPONSE ACCOMPANYING ABOLITION OF THE CENTRAL NERVOUS SYSTEM\*

EDWARD C MASON, M D, PH D, AND PHILIP HITCHCOCK, M S  
OKLAHOMA CITY OKLA

COHNHEIM and Roy\* (1883) first demonstrated the vasoconstriction produced in the kidney by stimulation of the splanchnic nerve Bradford<sup>3</sup> (1889) repeated this observation and also showed that stimulation of the nerve at a low frequency (about 1 per second) produces dilatation rather than constriction of the kidney Bardier and Frenkel<sup>1</sup> (1899) studied the vasoconstrictor action of adrenal extract on the kidney, and, in two instances, observed a slight increase in volume

One of us (E C M), while working in the laboratory of Dr D E Jackson (1920), observed a marked increase in kidney volume following the destruction of the central nervous system and the administration of epinephrine Since the authors have been unable to find any reference in the literature to studies on the relationship between the destruction of the central nervous system and kidney volume response, we have repeated our observations and attempted to find an explanation for the reversal of kidney volume response under such conditions

### EXPERIMENTAL PROCEDURE

All of the experiments were performed on healthy dogs, ranging in weight from 7 to 20 kg They were anesthetized either with ether or with sodium pentobarbital (nembutal, Abbott), ether being used in the majority of the experiments If nembutal was used, it was injected intraperitoneally in doses of 1 gram per 5 pounds body weight, augmented by intravenous doses of one tenth the original when required

\*From the Department of Physiology University of Oklahoma School of Medicine Aided in part by a grant from the Research Appropriation of the University of Oklahoma School of Medicine

Preliminary report presented before the American Physiological Society Memphis Tenn April 23 1937

Received for publication September 22 1935

Blood pressure was recorded from the right carotid artery, using a mercury manometer attached by rubber tubing to a glass arterial cannula. The recording system was filled with 20 per cent sodium citrate. The Jackson kidney and spleen oncometers and recording tambours were used to record kidney and spleen volume changes. The kidney oncometer was usually placed on the left kidney since the right lies somewhat higher in the abdomen than the left, its pedicle is shorter, and it is usually bound by a heavy renohepatic ligament which must be torn loose, producing severe trauma to the kidney, and frequently partial decapsulation or rupture of the inconstantly present blood vessels to the kidney or adrenal gland.

Injections of epinephrine were made directly into a 2 cm. section of the right femoral vein, exposed just below the inguinal ligament.

*Destruction of the Central Nervous System.*—The scalp is slit in the midline from just anterior to the occipital protuberance to about 2 cm. behind the eyes, whence the incision is carried laterally behind the eye for 3 or 4 cm. The temporalis muscle is cut through, and the area of origination is scraped clean. Using a Galt trephine, a hole is cut through the skull and the button of bone is removed. With the handle of a scalpel the brain is thoroughly macerated. This procedure liberates brain tissue extract, which is an efficient hemostatic, preventing death from hemorrhage. The ether bottle is quickly disconnected, since anesthesia is not required after destruction of the central nervous system, and either a bellows or an artificial respiration pump is attached in its place. The animal's head is returned to its original position, and the pithing wire is introduced through the foramen magnum. The pithing wire is made of a single strand of 16 gauge copper wire bent back on itself and twisted. It is passed down the spinal canal with an interrupted, twisting motion, thoroughly macerating the spinal cord. The wire is then withdrawn, pushing it backward and forward throughout the extent of the spinal canal, and the trephine hole is plugged with a gauze sponge. If pithing is complete, the blood pressure will fall to about 40 mm. Hg and maintain a constant level.

#### KIDNEY VOLUME CHANGES IN RESPONSE TO EPINEPHRINE

*Normal Response.*—In the normal animal the kidney volume reaction to intravenously administered epinephrine is entirely, or almost entirely, a reduction in volume. Fig. 1 is the record obtained from an unpithed dog, weighing 11.8 kg., anesthetized with ether. The top line is kidney volume, the middle line is blood pressure, the lowest is time in ten-second intervals and the base line for blood pressure. At the point marked (X), 0.3 c.c. of epinephrine at a concentration of 1:10,000 was injected intravenously. It will be observed that the kidney volume curve shows a marked fall during the time of the rapid rise in blood pressure, a period during which the constriction was maintained, corresponding to the maximal elevation of blood pressure, and a return to nearly normal volume. Inasmuch as the only contractile element of any importance in the kidney is the musculature of the blood vessel walls, it follows that any changes in kidney volume must be due to changes in the caliber of the vascular channels (Herrick, Essex, and Baldes, 1932').

There is a deviation from this reaction which occurs occasionally. As shown in Fig. 2 there is sometimes a slight increase in the volume of the kidney accompanying a slight fall in systemic blood pressure before the principal fall in kidney volume and rise in blood pressure occur. Fig. 2 shows not only the curves of carotid blood pressure and kidney volume, but also the record of spleen volume as well. The preliminary fall in blood pressure and rise in kidney volume is probably due to active vasodilatation.

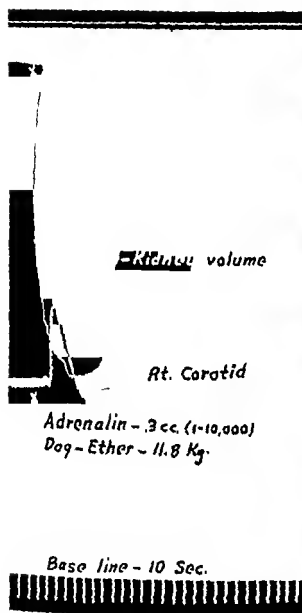


Fig. 1.



Fig. 2.

*Kidney Volume Reaction in Pithed Animal*—The kidney volume reaction to intravenously administered epinephrine is altered by pithing, as is demonstrated in Fig. 3, a record obtained from a dog weighing 7 kg. The animal was operated upon under ether, the brain and spinal cord were pithed completely, and artificial respiration was instituted. The upper curve is kidney volume, the middle line carotid blood pressure, and the lowest the base line and time record. It will be observed that the blood pressure is much smoother than that in the unpithed animal and that the blood pressure reaction to 0.5 c.c. of epinephrine (1:10,000) is a precipitous rise, the maximum being approached more quickly than in the normal.



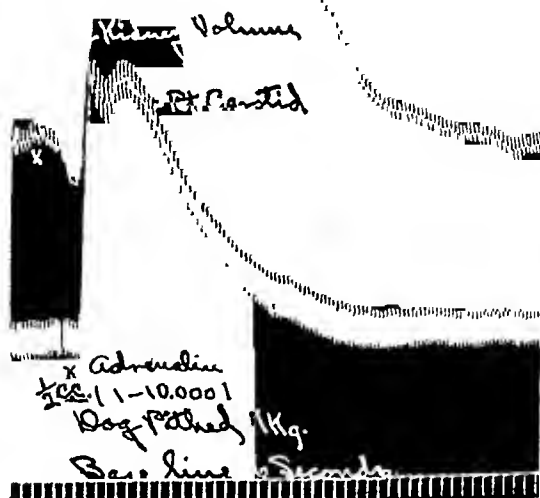


Fig. 3.

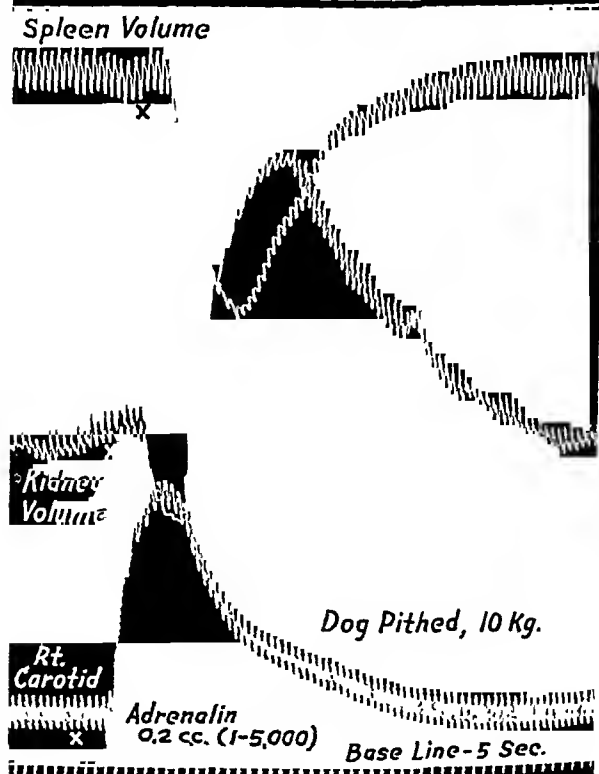


Fig. 4.

The kidney volume curve is characterized by, first, a fall of much smaller extent than the normal, a short period during which the constriction is maintained, and then an increase in volume to a level far above the normal. The ascending limb of the curve begins at about the time the blood pressure reaches its maximum. The final phase is a descent to normal, the kidney volume falling more rapidly than the blood pressure, so that the two reach basal levels at about the same time.

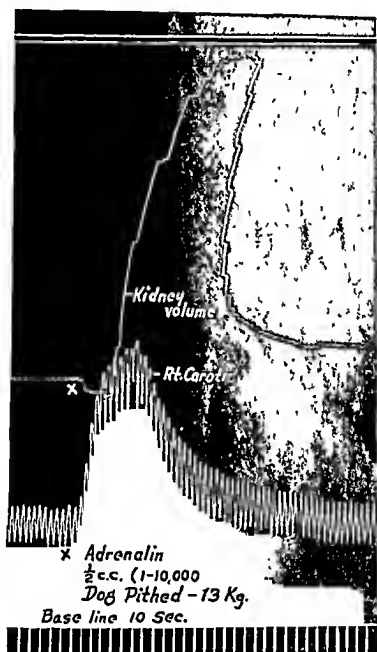


FIG 5

Fig 4 is presented in order to illustrate the difference in response to adrenalin before pithing and after pithing. This tracing was obtained from the same animal as Fig. 2. It will be observed that the spleen volume decreased in both tracings, but that the kidney volume response was just the reverse of that observed in Fig. 2. It is probable that the various other organs do not respond as the kidney does, by dilatation after destruction of the central nervous system, for if such response were generalized, there would of necessity be a drop in blood pressure with such generalized organ dilatation.

Apparently the anesthetic used was without marked effect on the responses obtained, since Figs. 2 and 4 were obtained from an animal anesthetized with

sodium pentobarbital, and the remaining Figs. 1, 3, 5, and 6 were obtained from animals anesthetized with ether. While there may be some difference in the degree of response produced, the type of curve is approximately the same.

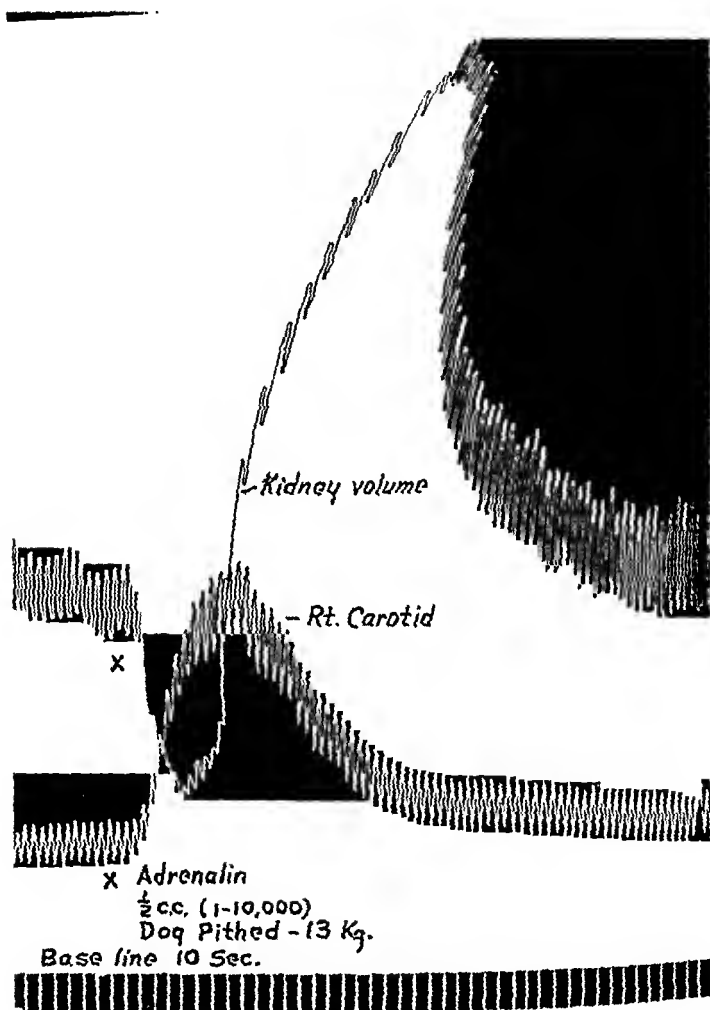


Fig. 6.

#### DEDUCTIONS

In searching for an explanation for this reversal of kidney volume response following destruction of the central nervous system, we are forced to consider the following:

*First: The obstruction of the venous return from the kidney.* It is evident that any mechanical obstruction of the renal veins, such as might be produced by faulty application of the kidney oncometer, would interfere with the return circulation, and thus cause an increase in the size of the kidney as a response to increased blood supply. This factor may be ruled out in our experiments, since analysis of our results show that (1) the return kidney volume to normal accom-

pauzes the blood pressure curve, (2) we have repeatedly obtained the same type of curve from the same animal, reproducing it as often as fifteen times, (3) animals, such as the one producing records (2) and (4), gave a decrease in kidney volume response before pithing and an increase following destruction of the central nervous system

*Second Possible shocklike condition of the animal with an accompanying state of stimulation of the vasomotor system* If this explanation is offered it must be considered as specific for the kidney for it will be recalled that the spleen does not share the volume reversal after destruction of the central nervous system. Also, if all other organs responded as the kidney does the blood pressure would not show the sudden and marked rise recorded. The best results are observed when the oncometer is speedily and easily attached and when pithing is done quickly, in other words, the best results are obtained with good not faulty, technique

*Third. Direct stimulation of vasodilator nerve endings* Bardet and Frenkel<sup>1</sup> on two occasions observed vasodilation in the renal vessels after small doses of the extract of the adrenal medulla. Biberfeld<sup>2</sup> and Ogawa<sup>3</sup> confirmed these results, although Elliott<sup>4</sup> was unable to do so. Also, it has been repeatedly demonstrated that the kidney will respond to epinephrine or stimulation of the splanchnic nerve by dilatation after the administration of ergotoxine or ergotamine. The following two points suggest that the increase volume response of the kidney is not due to the stimulation of the vasodilator nerve endings. (1) The threshold of the vasodilator is low, responding to minute doses of epinephrine, the concentration used to demonstrate vasodilating action usually being 1:100,000 in doses of 0.1 to 3 cc. Our experiments were performed using concentrations of 1:5,000 and 1:10,000 in doses ranging from 0.1 to 2 cc. (2) Cannon and Lyman<sup>5</sup> found that epinephrine did not produce vasodilation when the blood pressure fell below a certain minimal level, which they placed at 50 mm Hg, although it can be produced at such a blood pressure level if ergotoxine is administered

*Fourth Manipulation of the kidney damaging the vasoconstrictors of the kidney* This possibility is ruled out by (1) the record obtained on a normal intact animal showing a decrease in kidney volume while the same animal, without additional manipulation of the kidney, showed a reversal of kidney volume after destruction of the central nervous system, (2) the best results are obtained when the manipulation and kidney damage are the least

*Fifth The possible destruction of vasoconstriction mechanisms low in the cord* The possibility of destruction of subsidiary vasoconstrictor centers in the cord must be considered. The destruction of these centers, which are possibly in balanced opposition to the vasodilators of the kidney, would permit adrenalin to act on the vasodilators unopposed. However, it is difficult to conceive how such a mechanism would involve the kidney and not the other organs, such as the spleen

*Sixth Passive dilation produced by the increased blood pressure* In all cases the kidney responds in the pithed animal by an initial slight decrease in volume followed by a marked increase in volume, the maximum volume of the

kidney corresponding to maximum of the blood pressure. The two, kidney volume and blood pressure, then decrease simultaneously and reach the basal level together. This would be the sequence in passive dilatation of the kidney.

### CONCLUSIONS

1. The kidney volume response to epinephrine injections is changed by the destruction of the central nervous system, the response being essentially a reversal of that observed in the intact animal.

2. The increase in kidney volume accompanies the increase in blood pressure; therefore, the response might be a passive dilatation of the kidney.

3. There is also a possibility that destruction of subsidiary vasoconstrictor centers in the cord allows epinephrine to stimulate the unopposed dilatating fibers of the kidney.

### REFERENCES

1. Bardier, E., and Frenkel, H.: *Action de l'extrait capsulaire sur la diurese et la circulation renale*, J. de physiol. et de path. 1: 950, 1899.
2. Biberfeld, J.: *Beiträge zur Lehre von der Diurese xiii ueber die Wirkung des Suprarenins auf die Harnsekretion*, Arch. f. d. ges. Physiol. 119: 341, 1907.
3. Bradford, J. Rose: *The Innervation of the Renal Blood Vessels*, J. Physiol. 10: 353, 1889.
4. Cannon, W. B., and Lyman, Henry: *The Depressor Effect of Adrenalin on Arterial Pressure*, Am. J. Physiol. 31: 376, 1913.
5. Cohnheim, Julius Friedrich, and Roy, Charles S.: *Untersuchungen über die Circulation in den Nieren*, Arch. f. Path. u. Physiol. 92: 424, 1883.
6. Elliott, T. R.: *The Action of Adrenalin*, J. Physiol. 32: 401, 1905.
7. Herrick, J. F., Essex, H. E., and Baldes, E. J.: *Observations on Flow of Blood of Kidney*, Am. J. Physiol. 99: 696, 1932.
8. Ogawa, S.: *Beiträge zur Gefäßwirkung des Adrenalin*, Arch. f. exper. Path. u. Pharmacol. 67: 89, 1912.

## A NEW APPARATUS FOR OXYGEN THERAPY\*

CHARLES F. LOMBARD, A.B., AND CLARENCE NELSON, M.D.  
LOS ANGELES, CALIF.

OXYGEN therapy is often lifesaving when properly administered. However, the initial cost of therapeutic equipment, the expense of operation, the discomforts to the patient as well as lack of safety, inconvenience, and many more factors, contribute to the all too frequent denial or improper administration of oxygen therapy. In order to overcome these and other objections, various types of apparatus have been developed and tested on the wards of the Los Angeles County General Hospital. The equipment described below has been the most acceptable and has been in use since December, 1935. Satisfactory therapeutic results are obtained with a flow of 4 to 8 liters of oxygen per minute. This compares favorably with flow rates used with tents, nasal catheters, and other economical methods now in use. In the following discussion data will be introduced showing the effectiveness of this apparatus

\*From the Pathological Laboratory and the Service of Dr. Roy Thomas of the Los Angeles County General Hospital, Los Angeles.

Received for publication, August 22, 1938.

in increasing the alveolar oxygen tension of normal individuals. The clinical effectiveness of this apparatus will be included in a later report. It may be noted here that the results from the study of normal individuals have been amply substantiated on the wards of the Los Angeles County General Hospital, where approximately one million cubic feet of oxygen are given yearly by this method.

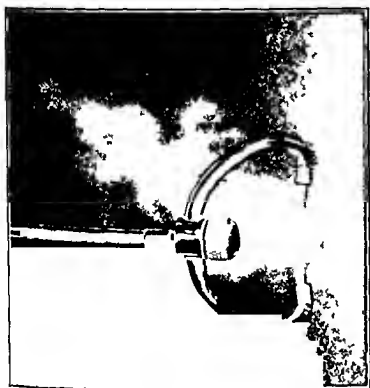


Fig. 1



Fig. 2

#### APPARATUS

The apparatus is an inhaler which is essentially a tubular Y constructed of metal or durable material. Gas issues from the curved arms of the Y in such directions that the streams meet and neutralize each other's velocities with the exception of a slightly resultant vector directed toward the center of the patient's upper lip. A high concentration of oxygen is held in the region most effective for inhalation, but without the irritating effect of a direct jet against the skin. This principle is shown in Fig. 1 using smoke in place of oxygen. The ordinary form of the inhaler has the stem portion as shown in Fig. 2, and is attached to the patient's forehead by means of adhesive tape (see Fig. 3), head band, or spectacle frame.

To increase the efficiency, a mask of cellulose acetate or similar material, made preferably in two sections, is pivotally mounted by means of eyelets on the ends of the fork (see Fig. 2). The mask in two sections allows the patient to have access to his mouth without removal of the equipment.

\*The mask in this figure is the large mask referred to later in the table.

kidney corresponding to maximum of the blood pressure. The two, kidney volume and blood pressure, then decrease simultaneously and reach the basal level together. This would be the sequence in passive dilatation of the kidney.

### CONCLUSIONS

1. The kidney volume response to epinephrine injections is changed by the destruction of the central nervous system, the response being essentially a reversal of that observed in the intact animal.

2. The increase in kidney volume accompanies the increase in blood pressure; therefore, the response might be a passive dilatation of the kidney.

3. There is also a possibility that destruction of subsidiary vasoconstrictor centers in the cord allows epinephrine to stimulate the unopposed dilating fibers of the kidney.

### REFERENCES

1. Bardier, E., and Frenkel, H.: *Action de l'extrait capsulaire sur la diurese et la circulation renale*, J. de physiol. et de path. 1: 950, 1899.
2. Biberfeld, J.: *Beiträge zur Lehre von der Diurese* xiii ueber die Wirkung des Suprarenins auf die Harnsekretion, Arch. f. d. ges. Physiol. 119: 341, 1907.
3. Bradford, J. Rose: *The Innervation of the Renal Blood Vessels*, J. Physiol. 10: 375, 1889.
4. Cannon, W. B., and Lyman, Henry: *The Depressor Effect of Adrenalin on Arterial Pressure*, Am. J. Physiol. 31: 376, 1913.
5. Cohnheim, Julius Friedrich, and Roy, Charles S.: *Untersuchungen über die Circulation in den Nieren*, Arch. f. Path. u. Physiol. 92: 424, 1883.
6. Elliott, T. R.: *The Action of Adrenalin*, J. Physiol. 32: 401, 1905.
7. Herrick, J. F., Essex, H. E., and Baldes, E. J.: *Observations on Flow of Blood of Kidney*, Am. J. Physiol. 99: 696, 1932.
8. Ogawa, S.: *Beiträge zur Gefäßwirkung des Adrenalin*, Arch. f. exper. Path. u. Pharmacol. 67: 89, 1912.

## A NEW APPARATUS FOR OXYGEN THERAPY\*

CHARLES F. LOMBARD, A.B., AND CLARENCE NELSON, M.D.  
LOS ANGELES, CALIF.

OXYGEN therapy is often lifesaving when properly administered. However, the initial cost of therapeutic equipment, the expense of operation, the discomforts to the patient as well as lack of safety, inconvenience, and many more factors, contribute to the all too frequent denial or improper administration of oxygen therapy. In order to overcome these and other objections, various types of apparatus have been developed and tested on the wards of the Los Angeles County General Hospital. The equipment described below has been the most acceptable and has been in use since December, 1935. Satisfactory therapeutic results are obtained with a flow of 4 to 8 liters of oxygen per minute. This compares favorably with flow rates used with tents, nasal catheters, and other economical methods now in use. In the following discussion data will be introduced showing the effectiveness of this apparatus.

\*From the Pathological Laboratory and the Service of Dr. Roy Thomas of the Los Angeles County General Hospital, Los Angeles.

Received for publication, August 22, 1938.

loose contact between the patient's face and the mask. This looseness though it must not be extreme, is a necessary part of the system and plays an important role in eliminating a closed system and the rebreathing of metabolites.

The economy of the inhaler in oxygen use, low original cost and elimination of especially trained operators together with its effectiveness in oxygen therapy, has made it possible to adequately supply the needs of many patients where formerly only a few of these patients could be given this care.

#### SUMMARY

- 1 A new apparatus for oxygen therapy is described.
- 2 Therapeutic concentrations of oxygen are obtained on comparatively low oxygen flows.
- 3 The apparatus is convenient, inexpensive and comfortable.

We wish to express our appreciation to Dr. C. H. Thielen and Dr. D. R. Drury of the University of Southern California School of Medicine for their advice and guidance.

## EFFECTS OF SMOKING ON BLOOD SUGAR AND PERIPHERAL TEMPERATURES OF CONVALESCENTS\*

H. B. CATES, M.D., AND J. G. GIOVANNASZI, M.D., LOS ANGELES, CALIF.

INITIAL interest was centered upon whether there is a correlation between changes in skin temperatures and rise in blood sugar induced by cigarette smoking. As the study progressed, however, the observations made upon the convalescent proved the more important. Then negligible response to smoking prompted us to test several volunteer medical students, and these had vascular responses to smoking, similar to the reactions observed by other smokers.

The capillary blood sugar level of fasting nondiabetic patients has been determined by Nielsen<sup>1</sup> to be quite uniform and without oscillations. On a uniform diet the degree of variance of fasting blood sugar values was not over 10 mg per 100 cc. Working with fasting adults and recording respiratory quotients and blood sugar levels, Haggard and Greenberg<sup>2</sup> reported respiratory quotients between 0.78 and 0.82. The blood sugar values ranged from 0.08 to 0.10 per cent. Under identical conditions after smoking there was a rise of the blood sugar above 0.13 per cent. This rise is accredited to an increased adrenalin output possibly due to nicotine stimulation. However, Dill, Edwards, and Forbes<sup>3</sup> made observations upon the capillary blood sugar level in 10 subjects after smoking one cigarette and found that nine tenths of the subjects were within 5 per cent of the control level. There was no significant trend. In addition, lactic acid values were not appreciably altered by smoking. They conclude that carbohydrate metabolism is not changed by cigarette smoking.

\*From the Department of Medicine of the University of Southern California.  
Received for publication September 26, 1938.







There is an apparent agreement of reports on the lowering of peripheral temperature of subjects after smoking. Maddock and Coller<sup>4</sup> find a fall of  $5.9^{\circ}$  C. of the fingers and  $0.50^{\circ}$  C. of the toes while smoking.

Barker,<sup>5</sup> testing a group of 20 patients, found a significant drop of  $1.8^{\circ}$  C. after smoking three cigarettes in rapid succession. Five patients had temperature changes in both fingers and toes. Johnson and Short,<sup>6</sup> when comparing smokers with nonsmokers, reported that 88 per cent of smokers showed an average drop of  $2.5^{\circ}$  C., while with nonsmokers only 34 per cent showed a drop, the average being  $1.8^{\circ}$  C. This is attributed to the fact that the latter did not inhale. Wright and Moffat<sup>7</sup> conclude that practically all subjects who inhale will show a temperature drop, though not necessarily every time the test is taken. An individual may have a lowering in skin temperature of  $5.5^{\circ}$  to  $6.6^{\circ}$  C. upon one day and as little as  $0.6^{\circ}$  to  $1.7^{\circ}$  C. with the same tobacco product upon the next day. Apparently in 16 out of 50 tests the subjects complained of mild toxic symptoms. This was manifest by tingling of the fingers and slight vertigo. In 7 instances nausea, vomiting, cold sweats, and syncope occurred. In another report, Maddock, Malcolm, and Coller<sup>8</sup> state that cigarette smoking produces equivalent decrease in skin temperature of women and men. They find more marked changes in Jewish subjects than in gentiles. They also note increase in blood pressure and pulse rate in all of the subjects tested. Their protocols show that 28 out of 29 patients were in the third decade of life and that 15 cases out of 29 had increases of systolic pressure which were less than 15 mg. mercury. Eight of the 29 had temperature reduction of either fingers or toes which was less than  $1.5^{\circ}$  C. Herrell<sup>9</sup> reports an idiosyncrasy to tobacco in a man 57 years of age, whose chief complaint was periods of rather severe dizziness. After smoking a cigarette the blood pressure was found to rise from 118/70 to 180/110, while the peripheral temperature, tips of fingers, decreased on an average of  $1^{\circ}$  to  $1.5^{\circ}$  C. between the first and second readings. Herrell and Cusick<sup>10</sup> reported 3 more sensitive subjects who reacted to smoking with elevation of blood pressures and associated spasm of the retinal vessels. This response is regarded as one of idiosyncrasy to tobacco.

#### PROCEDURE

All of the subjects studied, with exception of 6 students, were patients selected from a medical ward of the Los Angeles General Hospital, with the requirement that they were afebrile and received no medication except digitalis. The majority of these had either hypertension or heart disease, for with these, it was believed temperature and blood sugar fluctuations might prove more marked than in other diseases. It must be emphasized that no patient was urged to smoke more than was his custom, and similarly no untoward effect was reported by the subject.

The age incidence of the subjects varied from the third to the seventh decade. All were exposed for an hour or more to a constant temperature. Tests were made at a fixed temperature between the extremes of  $70^{\circ}$  and  $80^{\circ}$  F. The room temperature oscillations in the majority of instances were within one degree. Fasting capillary blood sugars were determined by the colorimetric micromethod of Benedict. A control specimen, the second fifteen

minutes, and a third, thirty minutes later, were taken. A Taylor's dermometer was used, and the temperature of the tips of the first three fingers and toes was read for changes in conjunction with the blood sugar tests. Upon others, records were made of the temperature changes of the fingers and toes of the right side. In addition, the pulse rate and blood pressures were determined. The pressures were taken with a mercury sphygmomanometer upon the left arm. The apical heart rate was counted for a period of thirty seconds. Temperature determinations were made at five minute intervals, allowing at least twenty minutes to establish a control of the peripheral temperature before requesting the subject to begin smoking. Cigarettes of the popular brands were offered, and the subject consumed from two to five without interruption. About two thirds of each cigarette was consumed.

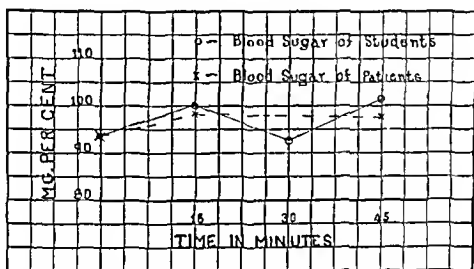


Fig 1—Relationship between the capillary blood sugar levels before and during cigarette smoking

### RESULTS

The lowering of peripheral temperature during smoking tests made upon 6 medical students was pronounced. The least decrease was  $1.8^{\circ}\text{C}$ , and the maximum,  $6.5^{\circ}\text{C}$ . Fluctuations of skin temperature under standard conditions which were less than  $1.5^{\circ}\text{C}$  were not regarded as significant and were not ascribed to tobacco<sup>11</sup>. The pulse rate counted at five minute intervals was increased by smoking, with a maximal increase of 32 and a minimum of 12 beats per minute. Blood pressure was elevated in most of the young subjects. The second subject had a systolic elevation of 35 and a diastolic increase of 25. The least change, noted in the fifth subject, was 13 systolic and 4 diastolic. These findings compare well with many previous reports, but serve to contrast the paucity of fluctuations noted upon the convalescent hospital patients.

The hypertensive group of patients while smoking showed no greater variation of skin temperature than occurred during the preceding control period of forty five minutes. In this group, highest blood pressure rise was 15 mm, the average 8 mm. This is to be contrasted with figures in the student group when the highest was 35 mm and the average rise was 19 mm.

The third group, those patients having normal blood pressures, and the majority of whom were admitted to the hospital suffering cardiac decompensation gave a negative temperature response. Of 24 studies only 4 had a

temperature decrease of their toes over  $1.5^{\circ}\text{C}$ . Two of 12 had depressed finger temperatures.

A composite curve representing 26 curves of capillary blood sugar determinations on 20 subjects, establishes no elevation of the fasting dextrose values after smoking two to five cigarettes. Artificial elevation of the blood sugars to 400 mg. per cent by intravenous injection of 50 c.c. of 50 per cent glucose likewise had no indirect effect upon peripheral temperature.

#### DISCUSSION

At present clinicians have no unanimity of opinion as to whether smoking is detrimental to patients suffering from certain diseases. Abstinence from tobacco is considered by some physicians to be chiefly responsible for the alleviation of symptoms in Buerger's disease, hypertensive heart disease, and peptic ulcer. The conclusion of Thienes and Butt,<sup>12</sup> after making well-controlled animal experiments, is that no demonstrable circulation lesions may be attributed to chronic nicotine poisoning in the animals tested. On the basis of our observations we conclude that smoking affects the peripheral vascular system less in cardiac convalescents than in young healthy males who have not been confined to bed. Again, the older the subject is, the less susceptible his vascular tone. Results obtained from the subjects of the second and third decade may not by inference be applied to later decades of life.

#### CONCLUSION

1. Convalescents show less response to tobacco than young healthy adults.
2. Smoking does not elevate capillary blood sugars, and there is no relationship evident between blood sugar levels and peripheral temperatures.
3. Patients having hypertension show no more vascular response to smoking than the nonhypertensive.

#### REFERENCES

1. Nielsen, O. J.: On Oscillations of Blood Sugar Values Within Brief Periods, and the Blood Sugar Curve on Uniform Ingestion of Glucose, *Biochem. J.* 22: 1490, 1928.
2. Haggard, H. W., and Greenberg, L. A.: The Effects of Cigarette Smoking Upon the Blood Sugar, *Science* 79: 165, 1934.
3. Dill, D. B., Edwards, H. T., and Forbes, W. H.: Tobacco Smoking in Relation to Blood Sugar, Blood Lactic Acid and Metabolism, *Am. J. Physiol.* 109: 118, 1934.
4. Maddock, W. G., and Collier, F. A.: Peripheral Vasoconstriction by Tobacco Demonstrated by Skin Temperature Changes, *Proc. Soc. Exper. Biol. & Med.* 29: 487, 1932.
5. Barker, N. W.: Vasoconstrictor Effects of Tobacco Smoking, *Proc. Staff Meet., Mayo Clin.* 8: 284, 1933.
6. Johnson, H. J., and Short, J. J.: Effect of Smoking on the Skin Temperature, *J. Lab. & Clin. Med.* 19: 962, 1934.
7. Wright, I. S., and Moffat, Dean: The Effects of Tobacco on the Peripheral Vascular System: Further Studies, *J. A. M. A.* 103: 318, 1934.
8. Maddock, W. G., Malcolm, R. L., and Collier, F. A.: Thrombo-angiitis Obliterans and Tobacco: The Influence of Sex, Race, and Skin Sensitivity to Tobacco on Cardiovascular Responses to Smoking, *Am. Heart J.* 12: 46, 1936.
9. Herrell, W. E.: Idiosyncrasy to Tobacco: Report of a Case, *Proc. Staff Meet., Mayo Clin.* 13: 1, 1938.
10. Herrell, W. E., and Cusick, P. L.: Vascular and Retinal Abnormalities Following Inhalation of Tobacco Smoke: Preliminary Report, *Proc. Staff Meet., Mayo Clin.* 13: 273, 1938.
11. Bordley, J., Grow, M. H., and Sherman, W. B.: Intermittent Blood Flow in the Capillaries of Human Skin, *Bull. Johns Hopkins Hosp.* 62: 1, 1938.
12. Thienes, C. H., and Butt, E. M.: Chronic Circulatory Effects of Tobacco and Nicotine, *Am. J. M. Sc.* 195: 522, 1938.

# LABORATORY METHODS

## A MANUAL OF NEUROHISTOLOGIC TECHNIQUE

OSCAR A. TURNER, M.D., NEW HAVEN, CONN.

*Contents*—I General Considerations II Stains for Nerve Cells III Stains for Myelin  
Sheaths IV Neurofibril Stains V Staining of Fat VI Nonmetallic Glia Stains VII  
Metallic Glia Stains VIII Connective Tissue Stains IX Pituitary and Pineal Stains  
X Spirochete Stains XI Miscellaneous Staining Methods XII Formulas Appendix  
References

### CHAPTER I

#### GENERAL CONSIDERATIONS

*Fixation of Tissues*—The importance of the immediate fixation of tissues after death cannot be overemphasized. Tissue which has been allowed to remain unfixed for some hours often gives indifferent and unsatisfactory results and is especially prone to artifact formation. This is particularly true in respect to the silver impregnation methods.

Although the methods selected here were primarily for use in a general pathologic laboratory where formalin is the routine fixative used reference will be made to certain special methods of fixation. Next to formalin Zenker's fluid is probably the most commonly employed tissue fixative and is particularly useful where many of the connective tissue and nonmetallic glial stains are to be used. Formalin ammonium bromide is necessary as a fixative for most of the original metallic glial methods although at the present time there are several modifications of these stains which give excellent results on formalin fixed material. Fixation in Regaud's solution or Zenker's solution is required for many of the more selective pituitary stains, although there are several satisfactory methods which may be employed on formalin fixed material. The present methods of treating tissue prior to staining makes possible the use of tissues fixed in formalin for the demonstration of practically every element of the nervous system.

\*From the Institute of Pathology and the Anatomical Laboratories of Western Reserve University, Cleveland.

Dr. Harry Goldblatt, Dr. Howard T. Karsner, and Dr. Albert Steegmann of the Institute of Pathology and the late Dr. T. Wingate Todd of Yale University have freely of their time and experience and proved to be a source of a debt of gratitude for their helpful criticism. The various members of the technical department, Miss Irene Boten, Mr. Donald Burt, and Miss Emily Squire were patient in my demands upon their time and experience. Dr. Harry Zimmerman of the Department of Pathology, Yale University, was kind enough to review the methods and offer valuable advice. Finally, thanks are due to Miss Edith Orling and Miss Frances Turner who did much of the technical work in the preparation of the manuscript. With all this assistance any errors that have crept into the text can only be mine.—Oscar A. Turner, M.D.

This article will appear in several installments. A list of selected references will appear in the last installment.

*Removal of Tissue.*—The method of removing blocks of tissue depends upon the nature and location of the lesion to be studied. For routine study of the brain, Spielmeier recommends taking blocks of tissue from:

1. Posterior part of the first frontal convolution.
2. Both central convolutions, approximately at the borderline of the upper and middle thirds.
3. First temporal convolution.
4. Region of cornu ammonis.
5. Region of the calcarine tissue.
6. Middle of the basal ganglia together with the island of Reil.
7. Posterior portion of the basal ganglia with the island of Reil.
8. Region of the oculomotor nuclei.
9. Inferior olives.
10. Vermis and cerebellar hemispheres.

11. Spinal cord segments: cervical and lumbar enlargements, upper cervical, upper and lower dorsal and sacral regions. Additional blocks will depend upon the nature of the individual specimen to be studied. For the study of tumors, blocks should always include a considerable portion of the adjacent nervous tissue and at least half the diameter of the tumor if this is feasible. Several blocks of tissue should be taken from various portions of the larger tumors, and a complete cross section is recommended for the smaller growths. Longitudinal, as well as cross sections of peripheral nerves, are often found useful. The blocks of tissue should not exceed 5 mm. in thickness and should be fixed in at least ten times their volume of fixative. The size of the tissue block, other than thickness, depends upon the individual lesion to be studied, the ability of the technician to handle very large or very small sections, and the facilities at hand for the preparation of very large sections.

*Laboratory Equipment.*—Special equipment, other than that found in the average laboratory for histologic technique, is not necessary for the staining of the nervous system. Small glass dishes, holding 30 to 40 c.c., have been found useful for frozen section work. Where larger receptacles are needed, small beakers or large Petri dishes may be used. Glass rods bent to form a hook are used for handling frozen sections, and a small camel's-hair brush is employed to draw these on the glass slide. Bibulous paper is best for blotting sections, although filter paper may be used.

*Gelatin Embedding.*—For many methods in which paraffin embedding is contraindicated or not feasible, it is necessary to embed the tissue in gelatin before making frozen sections. The following method has been found to give excellent results. Blocks of formalin-fixed tissue are washed in running water for twenty-four hours, after which they are placed in thin gelatin solution (12 per cent) for a similar length of time in the 37° C. incubator. They are then transferred to thick gelatin solution (25 per cent) and allowed

to remain twenty-four hours at the same temperature. The blocks are then placed in small dishes and allowed to harden in the thick gelatin for one to two hours in the icebox. The excess gelatin is then trimmed off, and the blocks are further hardened in 10 per cent formalin for one to two days and washed for one to two hours in running water before sectioning. The blocks are sectioned on the freezing microtome in the usual manner. The method is used in making frozen sections of small fragments of tissue or where the tissue sections crumble and tear when prepared by the usual frozen section method.

The preparation of the gelatin solution is quite simple. A weighed amount of table gelatin is added to three times its amount of a 1 per cent aqueous solution of carbolic acid. This is allowed to remain in the incubator at 37° C until dissolved to form a thick fluid mass. To make the thin gelatin (12 per cent), dilute the 25 per cent solution with an equal part of the 1 per cent aqueous carbolic acid.

## CHAPTER II

### STAINS FOR NERVE CELLS

The most widely used methods for the demonstration of nerve cells and cortical architectonics are those based upon the principle devised by Nissl. Excellent results can be obtained upon formalin-fixed tissue, either embedded in eellodm or cut with the freezing microtome. The following modifications of the original method have been found to give consistent results with the minimum of effort. With a little practice differentiation can be earned to the point where the cell bodies and the Nissl substance stand out in sharp contrast to a colorless, or nearly colorless, background.

*Cresyl Violet Stain for Frozen Sections*—Blocks of tissue, not over 5 mm thick, are washed in running water for a half hour, after which they are allowed to remain in distilled water for about one hour. Frozen sections are cut at 15 microns.

1. Place sections in 50 per cent alcohol and allow to remain in the incubator at 37° C. for two days.

2. Transfer the warm sections to a 3.5000 aqueous solution of cresyl violet. (Filter stain through a single layer of very coarse filter paper before using.)

3. Heat until steam arises from the stain. Do not boil. Allow to cool.

4. Rinse sections in distilled water.

5. Transfer quickly to 70 per cent alcohol. Shake sections for about one minute.

6. Differentiate the sections in 96 per cent alcohol. This is the most important step and a little practice will enable one to determine how far the decolorization should be carried.

7. Pass sections through absolute alcohol for a few seconds to complete dehydration.



8. Clear in xylol. The sections should be held until the xylol has penetrated, so as to prevent agitation which may tear fragile sections.

9. Draw sections on a clean glass slide, blot with bibulous paper, cover with a drop or two of xylol, and mount in balsam. Cedarwood oil is better than balsam for mounting Nissl stains, but has the disadvantage of drying very slowly and imperfectly. Small weights should be placed on the cover slips to keep the sections flat while drying.

*Thionine Stain for Celloidin Sections.*—Sections of the celloidin-embedded tissue, cut at 15 microns, are placed in 70 per cent alcohol and kept in the incubator at 37° C. for twenty-four to forty-eight hours.

1. Transfer warm sections quickly to a 1:5000 aqueous solution of thionine.

2. Heat until steam arises, never allowing the stain to come to a boil. Allow to stand until cool.

3. Transfer to distilled water.

4. Quickly transfer sections to 70 per cent methyl (wood) alcohol and agitate for a few seconds.

5. Differentiate in 96 per cent methyl alcohol. (Methyl alcohol differentiates rapidly and is better suited for formalin-fixed tissue. Ethyl alcohol is less rapid and is used to differentiate alcohol-fixed material.)

6. Complete dehydration for a few seconds in absolute alcohol.

7. Clear in xylol.

8. Draw sections on a slide and after straightening, trim off the excess celloidin, blot quickly, and cover with balsam. Warm very gently and place cover slip over the section. Allow to stand about twenty minutes, heat gently again, and after pressing out the air bubbles, keep overnight under weights.

*Toluidine Blue Stain for Alcohol-Fixed Tissue.*—Although excellent results may be obtained from formalin-fixed tissue, stained either with cresyl violet or thionine, by far the best results are obtained through the use of tissue which has been fixed in 96 per cent alcohol and stained with toluidine blue.

Blocks of tissue not over 5 mm. thick are fixed for several days in at least ten times their volume of 96 per cent alcohol. The alcohol should be changed frequently. Following fixation, the tissue is embedded in celloidin and stained according to the method given above for celloidin-embedded tissue. However, a 1:1000 aqueous solution of toluidine blue is used for staining, and ethyl (grain) alcohol is used for differentiation instead of methyl (wood) alcohol. A 1:5000 aqueous solution of thionine may also be employed as a stain.

A more rapid, although less satisfactory, method may also be employed where a sliding microtome is available. After fixation the tissue is dried care-

fully between two sheets of filter paper and placed in its proper position on the wooden block. It is then covered with thin (12 per cent) celloidin and allowed to dry. The tissue is then sectioned on the sliding microtome at 20 microns, without further embedding. The sections are stained as for celloidin embedded tissue. The tissue may also be fixed to the wooden block with gum arabic and hardened in 96 per cent alcohol for a few minutes.

The following remarks apply to Nissl stains in general. If the differentiation is carried to the right degree, the background should be colorless or nearly so. The ganglion cells stand out clearly and are in sharp contrast to the background. If the sections are passed through chloroform after dehydration in the absolute alcohol, the blue color of the cells assumes a lilac cast, which gives considerable contrast. The Nissl substance takes a deep stain, and nuclear detail is clear. In the white matter the nuclei of the various glial cells take the stain deeply, but only occasionally can the suggestion of cell processes be made out. The walls of the larger vessels take the stain rather intensely, while in the cortex the capillaries can be identified with ease. Erythrocytes are stained deep blue but disintegrated blood often takes a greenish blue color. Pathologic pigments of various sorts usually take an intense blue, but not always so. Cellular lipid inclusions are not stained.

The stained sections have a tendency to fade and should be stored in the dark and never placed in direct sunlight. This is especially true of cresyl violet, which is very sensitive to sunlight. Sections stained as described above and kept in the dark have retained their color quite well for a number of years. It is best not to place the stained sections in an incubator.

Staining should be carried out in a large evaporating dish and differentiation in glass containers over a white background. Coloration of the celloidin in embedded material may be avoided by the use of fresh alcohols, especially the 95 per cent and the absolute. A 1 per cent aqueous stock solution of the dyes keeps for long periods and is easily diluted to the desired strength for staining.

### CHAPTER III

#### STAINS FOR MYELIN SHEATHS

Myelin sheath stains find their greatest use in general neuropathology, although their use in general pathology is by no means limited. The staining methods described below vary widely in the time required for staining, and all are easily carried out. With experience, there is little trouble in obtaining consistently reliable results.

*Pal Weigert Method*—This method is applicable to either frozen or celloidin sections. The tissue should be thoroughly fixed in 10 per cent formalin

1 Blocks of tissue, not over 1 cm thick, are placed in Weigert's primary mordant and allowed to remain for four to five days at room temperature. Weigert's primary mordant is prepared as follows

Potassium bichromate	5 gm
Chromium fluoride (fluorchrome)	2 gm
Water, to make	100 cc

2. The tissue is then transferred to Weigert's secondary mordant, where it is allowed to remain for twenty-four to forty-eight hours:

Copper acetate	5.0 gm.
Glacial acetic acid 36%	5.0 c.c.
Chromium fluoride	2.5 gm.
Water, to make	100.0 c.c.

Dissolve the chromium fluoride in boiling water, add the glacial acetic acid and copper acetate.

3. The tissue is dehydrated in alcohols of ascending strengths and embedded in celloidin in the usual manner. However, frozen sections may be cut at 30 or more microns and carried through the following procedures.

4. The sections are transferred to Weigert's hematoxylin and allowed to remain twenty-four to forty-eight hours. The hematoxylin is prepared as follows:

10% hematoxylin in absolute alcohol, ripened	10 c.c.
Water	90 c.c.

5. Wash until uniformly blue in water made alkaline by the addition of from 1 to 3 per cent of a saturated solution of lithium carbonate.

6. Differentiate in 0.25 per cent potassium permanganate until the gray matter is brownish yellow.

7. Transfer to the following solution:

Oxalic acid	1 gm.
Potassium sulfite	1 gm.
Water	200 c.c.

Allow to remain until the gray matter is colorless, or nearly so.

8. Wash in water and blue again in lithium carbonate solution.

9. Wash in water, dehydrate in alcohol, and clear in xylol. Mount in neutral balsam.

The myelinated nerve fibers appear dark blue to bluish black upon a colorless or slightly yellow background. The differentiation is sharp, and the finest nerve fibers are deeply stained. Cells and axis cylinders do not take the stain. Areas of demyelination appear pale yellow and devoid of stain, although where the process is incomplete, scattered nerve fibers may be seen.

In differentiating the sections, it is best not to allow them to remain in the potassium permanganate longer than one minute before washing and placing them in the oxalic acid solution. They should then be washed, blued in lithium carbonate solution, and again treated with the permanganate. This is to prevent the sections from assuming a yellowish-brown color, which will

resist decolorization by the oxalic acid. It is usually necessary to repeat this process several times before the correct degree of differentiation is obtained. The degree of decolorization can be carried out to suit the individual taste. The main disadvantages with this method lie in the length of time required for its completion and the fact that the tissue cannot be used for many other stains once it has passed through the Weigert mordants. The method has withstood the test of time, and is one of the most reliable.

The mordants retain their strength for considerable periods of time as does the oxalic acid solution. The potassium permanganate solution should be prepared as a 2 per cent stock solution and diluted just before use. The hematoxylin should be prepared from the alcoholic solution and also diluted just before use. It should not be used more than once.

*Spielmeyer's Method*—The method gives good results and can be completed in two days. Formalin fixed tissue cut with the freezing microtome is used. The tissue should be washed in running water for at least thirty minutes before sectioning to remove the formalin. Sections should be cut at 30 or more microns.

- 1 Allow frozen sections to remain in tap water for two hours before staining.

- 2 Transfer to 2.5 per cent iron alum solution (ferrous potassium sulfate) and allow to remain overnight. Care should be taken to see that the sections lie flat in this solution as there is a tendency for them to harden and wrinkles may be difficult to remove.

- 3 Rinse in distilled water and transfer to 70 per cent alcohol where they should remain ten minutes. Agitate during this time.

- 4 Transfer to an old hematoxylin solution made up as follows:

10% solution of hematoxylin in absolute alcohol	5 cc
Distilled water	100 cc

Spielmeyer states that if the hematoxylin solution is old and stains well, one to two hours is sufficient. It may be necessary to stain as long as six to ten hours. The sections should be uniformly black when removed from the stain. Inasmuch as there is a tendency for the sections to become extremely brittle in the hematoxylin staining, should not be prolonged more than is necessary.

- 5 Rinse in distilled water and differentiate in iron alum solution. Here it is well to use a 4 per cent solution for the early stages and a weaker solution 2.5 per cent or less toward the end. Sections should be handled carefully to prevent them from fragmenting. They may be transferred from one solution to another on slips of blotting paper.

- 6 Wash sections thoroughly in three changes of distilled water.

- 7 Dehydrate in ascending alcohols and clear in xylol. Mount in neutral balsam.

The results are similar to those of the Pal-Weigert method. Occasionally there is a slightly deeper yellow color to the background, but often this can be prevented by liberal washings where noted above. The hematoxylin solution may be used repeatedly if filtered before returning to the stock bottle, although some authors recommend using fresh hematoxylin solution each time. Its staining power increases with age. Washing the sections in 70 per cent alcohol removes fatty substances, and incomplete treatment at this stage results in uneven staining. If this occurs, the sections should be carried back through water to 70 per cent alcohol and again stained and differentiated. During differentiation the sections should be moved about, and for larger and deeply stained sections it may be necessary to replace the differentiating fluid with fresh solution. The disadvantage in this method lies in the extreme brittleness of the sections after removal from the stain, making it difficult to obtain intact preparations from large specimens.

*Weil's Rapid Method.*—This method may be used on material embedded in celloidin or paraffin or cut on the freezing microtome. Paraffin sections give less satisfactory results, but if used, should be cut at about 15 microns. Celloidin-embedded material gives the best results. When frozen sections are used, they should be carried through 95 per cent alcohol, absolute alcohol, xylol, and back down again through the descending concentrations of alcohol to water. The method is as follows:

1. Wash sections in distilled water.

2. Stain for ten to thirty minutes at 50° to 55° C. in a mixture of equal parts of (a) and (b). The stain should not be filtered and should not be used more than once. The individual components keep indefinitely.

(a) Ferric ammonium sulfate solution, 4%.

(b) 1% solution of hematoxylin which has been made by adding 90 c.c. of distilled water to 10 c.c. of a ripened 10% solution of hematoxylin in absolute alcohol.

3. Wash twice in tap water.

4. Differentiate in a 4 per cent aqueous solution of iron alum. For celloidin material, differentiate until the gray matter or areas of demyelination can be just distinguished; for paraffin sections, long enough to remove the stain from the back of the slide. Care should be taken not to overdifferentiate in iron alum, for in doing so the fine fibers lose their stain.

5. Wash in three changes of tap water.

6. Complete the differentiation in the following solution:

Borax	10.0 gm.
Potassium ferricyanide	12.5 gm.
Distilled water	1000.0 c.c.
This solution keep indefinitely.	

The decolorization should be controlled under the microscope

7 Wash in two changes of tap water

8 Wash for thirty seconds in dilute ammonia water (Add 6 drops of 28 per cent ammonia to 100 c.c. of water)

9 Wash in distilled water

10 Dehydrate in alcohol, clear in xylol, and mount in neutral Canada balsam

The above method has the advantage of being rapid and of giving fairly consistent results. The myelinated fibers are stained a deep blue and if the section has not been overdifferentiated in iron alum solution, the fine myelinated fibers can be traced with ease. The background is usually a light yellow color, and if care is taken to wash the tissues thoroughly after each stage of differentiation the color of the background is not too deep and the contrast is excellent. It is important that the hematoxylin used should be completely ripened, at least for six months. Failure to obtain staining of the tissue in the hematoxylin is usually due to the use of insufficiently ripened solution. The stain should not be filtered before use and it cannot be used more than once. For 10 celloidin sections cut at about 30 microns 50 to 75 c.c. of stain should be used. The length of time required for the sections to take the stain varies somewhat with the method used in sectioning the tissue. Celloidin embedded material should be left in the stain from twenty to thirty minutes, paraffin sections require ten to fifteen minutes. Large sections may become somewhat brittle, but never enough to cause serious trouble if the material is handled carefully. Large sections can be transferred from solution to solution on slips of blotting paper. It is not advisable to use paraffin sections if other tissue is available.

*Wolter Kulshitzky Stain for Myelin Sheaths*—This is the best of the various modifications of the original Weigert method for staining myelin sheaths. The method utilizes formalin fixed tissue which is embedded in celloidin. It has the disadvantage that, like the Weigert method, the tissue blocks must be mordanted before embedding, thus making them useless for other stains.

1 Place blocks of formalin fixed tissue directly into Weigert's primary mordant. They are left here in the dark for ten to fourteen days, depending upon the size of the block.

2 Wash in running water for one half to two hours.

3 Place in 70 per cent alcohol for one day.

4 Leave in 96 per cent alcohol for three to six days. Change the alcohol after the first day.

5 Leave in absolute alcohol for two days. Change alcohol after the first day.

6 Transfer tissue to absolute alcohol ether (1:1) and allow to remain for one to two days, the latter for the larger blocks of tissue.

7 Place in thin celloidin and allow to remain fourteen days or longer.

8. Transfer to thick celloidin for eight to fourteen days. The tissue must be kept in the dark up to the time the infiltration with celloidin is completed.

9. Embed in celloidin and section in the usual manner. Allow the blocks to harden for one day in 70 per cent alcohol before sectioning. The sections may be cut at from 25 to 100 microns, depending upon the purpose of the stain. Those cut at 25 to 30 microns may be used for detailed microscopie study, while those cut at from 50 to 100 microns are for low magnification or naked-eye examination.

10. The sections are received in 70 per cent alcohol. Transfer quickly through distilled water to Müller's fluid where they remain for eight days or longer.

11. Wash in two changes of distilled water and pass briefly through 70 per cent alcohol. Transfer to Kulsehitzky's hematoxylin and allow to remain overnight in the incubator at 37° C.

10% solution hematoxylin in absolute alcohol,	
ripened	10 c.c.
Glacial acetic acid	1 to 2 c.c.
Distilled water	100 c.c.
Heat together until dissolved.	

12. Wash in two changes of distilled water.

13. Leave in Müller's fluid for two minutes.

14. Transfer to tap water and wash for five minutes or longer. Change the wash water until it remains clear.

15. Transfer to 0.25 per cent aqueous potassium permanganate solution for ten to twenty seconds.

16. Transfer rapidly through distilled water into a mixture of equal parts of 1 per cent oxalic acid and 1 per cent potassium sulfate. Allow to remain about five minutes. Change solution when it turns brown.

17. Wash in several changes of distilled water. Return sections to the permanganate solution, wash and again decolorize in the bleaching mixture. Repeat this process until the desired degree of differentiation is obtained. Wash the sections thoroughly in distilled water and allow to remain overnight.

18. Dehydrate in ascending concentrations of alcohol, pass through carboxylol, and clear in several changes of xylol. Mount in balsam.

The myelin sheaths are stained a dark blue against a practically colorless background. The fine myelinated fibers are deeply stained, and the contrast is excellent. Poorly infiltrated tissue is difficult to section and fragments easily. The sections tend to become brittle in the hematoxylin, and Wertham suggests transferring them to pieces of paper, upon which they may be stained and differentiated with less danger of fragmentation. The method can be used on very large blocks of tissue if care is taken that the infiltration with celloidin is complete.

As with other myelin sheath stains employing ripened hematoxylin, the absolute alcohol solution must be at least six months old. Older hematoxylin gives better results. The 0.25 per cent potassium permanganate solution should be prepared fresh from a 2 per cent stock solution and like the bleaching mixture, should be changed frequently during the process of decolorization. The celloidin embedded blocks of tissue may be stored in 70 per cent alcohol. As in all frozen section or celloidin work small weights should be placed upon the cover slips after mounting until the balsam has hardened.

*March Method for Degenerating Myelin*—It is occasionally desirable, especially in tracing very small or scattered areas of degeneration, to use a method which gives a positive stain for the degenerated myelin. For this the March method, utilizing osmic acid as a staining agent is almost universally employed. Whereas in the previous methods the staining was limited to the sheaths of the uninjured myelinated nerve fibers, here the staining is confined to the myelin of the degenerated or partly degenerated fibers.

1 Small blocks of tissue, not more than 2 to 3 mm thick, are fixed in Muller's fluid or 3 per cent potassium dichromate, changing the solution every three or four days. The time of fixation varies from one to two weeks.

2 The tissue is transferred directly without washing into the following solution

Muller's fluid or 3 per cent potassium	
dichromate	2 parts
1 per cent osmic acid	1 part
Prepare fresh before use	

The tissue should be left in this solution for one to three weeks and the solution should be renewed weekly.

3 Wash stained blocks in running water for twenty-four hours.

4 Dehydrate as rapidly as possible and embed in celloidin.

5 Cut sections at from 10 to 5 microns according to purpose, and mount in dammar or balsam.

The degenerating myelin is seen as small black dots and globules on a light yellow or yellowish brown background composed of the unstained elements of the nervous system. The lipochrome granules of nerve cells may also be stained black or brown. The sections may be counterstained with saffranine, neutral red, or van Gieson's stain as desired. The length of time of fixation should not be unduly prolonged since overfixation may produce artifacts in the form of small black granules which are difficult to differentiate at times from degenerating myelin. It also should be noted that fat cells in the connective tissue stroma of nerves also take the black stain. The length of time required in the osmic acid mixture varies with the size of the blocks of tissue and the types of tissue being treated. The spinal cord and peripheral nerves usually require from eight to fifteen days, while twenty-one to twenty-eight days is generally sufficient for the cerebellum and cerebrum.



Anderson suggests the addition of 10 to 20 c.c. of 1 per cent acetic acid to each 100 c.c. of Marchi fluid to facilitate penetration. Material which has been fixed in formalin for not more than two to three days may be used by transferring directly to the osmic acid mixture. The results here, however, are less reliable than with tissue which has been fixed in Müller's fluid or 3 per cent potassium bichromate.

It is not possible to give exact figures for the time the tissue remains in many of the reagents. Not only do the time relationships vary with the size of the tissue blocks, but also with the portion of the nervous system from which the tissue was removed. The type of mammal, age of the tissue, and maturity of the tissue play a part as well. For this reason, wherever indicated, the time limits are given. In general, the time requirements are not rigid and good results are not difficult to obtain.

Precautions should be taken in the interpretation of the results of this method. Aside from the black particles of degenerating myelin, there may be an irregular precipitate which may be difficult to differentiate from actual degeneration. Care must be exercised in handling the unfixed tissue to prevent displacement of the degenerating myelin. Although it is best to dehydrate and embed the stained tissue block as rapidly as possible, it is also important that the process of dehydration be complete. Small pieces of tissue may be embedded in paraffin if chloroform and not xylol is used as a clearing agent.

Aside from the time element, the chief disadvantage in this method lies in the ease with which artifacts are produced. However, in experienced hands it is fairly reliable. For routine pathologic work any of the myelin sheath stains are to be preferred, and for experimental work it is best to control the results with one of the myelin sheath stains.

## CHAPTER IV

### NEUROFIBRIL STAINS

Staining methods for the demonstration of neurofibrils are important both to the study of general neuropathologic and tumor material.

*Bielschowsky's Silver Method for Neurofibrils.*—This method utilizes frozen sections cut at 10 microns from formalin-fixed tissue. The tissue should be washed in running water for one-half to one hour before sectioning, and the sections should remain in distilled water for two hours before staining. Paraffin-embedded material may be used also.

1. Transfer sections to 2 per cent silver nitrate solution and allow to remain for twenty-four to forty-eight hours. It is best to keep the sections in the dark while in this solution. They may be placed in 4 per cent silver nitrate in the incubator at 37° C. and left overnight to save time.

2. Rinse quickly in double distilled water.

3. Transfer sections to freshly prepared ammoniacal silver solution and allow to remain for ten to twenty minutes.

10% silver nitrate	5 cc
40% sodium hydroxide	5 drops

Wash the dark brown precipitate which is formed with three changes of double distilled water. Add water to make 5 cc and dissolve the precipitate by adding strong ammonium hydroxide drop by drop. Avoid an excess of ammonia. Dilute resultant solution to 20 cc with double distilled water.

The sections should be removed when they are deep brown in color.

4. Rinse rapidly in two changes of double distilled water, and reduce in 20 per cent formalin made up with tap water. Reduce for five to ten minutes. The sections should assume a slate gray or grayish black color.

5. Wash in double distilled water for ten minutes.

6. Tone sections in the following solution:

1% gold chloride	15 drops
Distilled water	10 cc

The toning solution may be acidified with two or three drops of glacial acetic acid. Treatment should be continued until the sections are a light reddish-violet color. It is preferable, however, to tone the sections for an hour in a very dilute solution of gold chloride, using 2 to 3 drops of 1 per cent gold chloride in 10 cc of distilled water. The 1 per cent gold chloride solution keeps for long periods in the dark.

7. Wash in distilled water.

8. Fix in 5 per cent sodium thiosulfate for one half to one minute.

9. Wash freely in distilled water. Mount on glass slide from 50 per cent alcohol blot carefully, complete dehydration in two changes of absolute alcohol, and clear in carbonyl (1:10). Mount in balsam.

There is excellent staining of the axis cylinders and dendrites as well as the intracellular neurofibrillar network. The latter appears as an interlacing web of delicate fibrils, dark brown or black depending upon the degree of toning in the gold bath. The axis cylinders are stained an intense black as are the dendrites. There is occasional faint impregnation of the glial cells and in tissue which is the seat of marked gliosis, impregnation of the glial fibers may be quite definite. The background is colorless or light purple depending upon the toning.

The silver nitrate solution should be made fresh just before use. Care should be taken in preparing the silver bath that an excess of ammonium hydroxide is not added. A small amount of very fine granular, black precipitate should be allowed to remain undissolved. At least a liter of double distilled water should be used in washing, decanting the fluid after allowing the precipitate to settle. The formalin used for reduction should be neutralized with calcium carbonate. Sections may be carried up to the point of

toning and then counterstained with the Alzheimer-Mann method for glial fibers. Herxheimer's method for fat is also a very useful counterstain.

*Cajal's Method for Neurofibrils.*—The following method of Cajal is taken from Spielmeyer and is said to give excellent impregnation of neurofibrils in the cerebellum. It has been found quite useful for tumor material.

1. Frozen sections, cut at 30 to 40 microns, from formalin-fixed tissue are washed rapidly in two changes of distilled water.

2. Place sections for two to twelve hours at room temperature in the following mixture:

2% silver nitrate	10 c.c.
Pyridine	8 drops
96% alcohol	5 to 6 c.c.

The sections are removed when they assume a light brown color. Placing them in an incubator hastens the reaction and assures better impregnation. Three to five hours is usually enough time.

3. Pass the sections rapidly through absolute alcohol.

4. Reduce without previous washing in the following solution:

Hydroquinone	0.3 gm.
Distilled water	70.0 c.c.
Neutral formalin	20.0 c.c.
Acetone	15.0 c.c.

The reduction is completed in one to three minutes.

5. Wash freely in distilled water.

6. Tone in gold chloride 1:500; wash in distilled water and fix in 5 per cent sodium thiosulfate for one to two minutes.

7. Wash thoroughly in distilled water. Mount on a glass slide from 50 per cent alcohol, blot carefully, complete dehydration in two changes of absolute alcohol, and clear in xylol. Mount in balsam.

The stain may be completed after reduction, washing freely in distilled water, fixing in 5 per cent sodium thiosulfate, washing, dehydrating, clearing and mounting as usual. This gives the neurofibrils a deep brown to black color against a golden-brown background.

There is intense impregnation of the nonmyelinated axis cylinders, especially in the cerebellum where the arborizations of the basket cells and the mossy and climbing fibers are clearly shown. These elements are stained black upon a light gray or purplish-gray background, the latter if gold chloride toning is employed after reduction.

The reducing solution keeps well in a tightly stoppered bottle. Passage of the sections through the absolute alcohol after impregnation in the silver bath should be done rapidly to prevent loss of silver. The method gives good results on tissue which has been fixed in formalin for several years.

(To be continued.)

## A SIMPLE BLOOD PIPETTE CLEANER\*

OSCAR BLOCH, JR., M.D. LOUISVILLE, KY

IN AN effort to lessen the drudgery of a busy clinical laboratory, the following device for simultaneously cleaning several blood pipettes was made

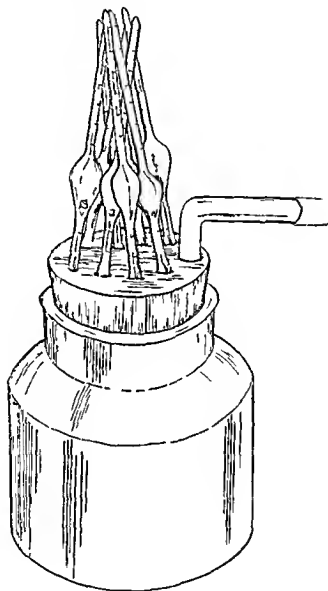


FIG 1

A wide mouthed (60 to 70 cc to one half pint) jar is fitted with a rubber stopper through which eight small (3 mm) holes in two straight lines, and one larger (6 mm) hole near the edge of the stopper are bored, as shown in the drawing. Into the larger hole is fitted a glass L tube connected by a rubber tube to a faucet suction pump. The eight holes, which are sloped toward one another on the outside of the bottle, receive the butt ends of the pipettes.

\*From the Department of Pathology, University of Louisville, Louisville, Ky.  
Received for publication June 17, 1938.

In use, the holes are loaded with pipettes, and the suction is turned on. The bottle is then inverted successively over the distilled water, alcohol, and ether bottles, so that the tips of the pipettes dip into these fluids. Waste fluid entering the wash bottle is drawn off by the suction tube, whose inner end lies flush with the inner surface of the stopper and is held downmost in use. If all eight holes are not needed, the unoccupied ones can be covered by the finger.

The pipettes are dried by merely setting the bottle down on the table, the hands then being free for other tasks. If one wishes, the ether wash can be omitted, and the drying done for several minutes after the alcohol.

---

### SMALL ANIMAL HOLDERS FOR INTRAVENOUS INJECTION\*

---

K. K. CHEN, PH.D., M.D., AND CHARLES L. ROSE, A.B., INDIANAPOLIS, IND.

---

**I**NTRAVENOUS injections in rodents are no novelty to pharmacologists and physiologists, but satisfactory animal holders seem to be wanting. In the late Professor A. S. Loevenhart's laboratory at the University of Wisconsin, a mailing case was used for injections into one of the tail veins of rats—the bottom of the case being perforated, and the lid being replaced by a cork with a hole at the edge to accommodate the tail. This simple device usually served the purpose. Its chief disadvantages are, however, retraction of the tail by small animals and difficulty in cleaning the cardboard case. With the aim of improving this apparatus, we have devised metal holders† for mice, rats, and guinea pigs, and found them so convenient that they may be suitable for other investigators.

The rat holder as shown in Fig. 1 consists of two hollow cylinders, one inside the other. The outer cylinder measures 9.4 cm. in length, and 5.2 cm. in outside diameter, its thickness being 0.25 cm. all around. One end of it is closed with a perforated plate. The holes are evenly distributed, 0.65 cm. in diameter, and 7 in number—adequate for ventilating purposes. The inner cylinder is telescoped into the outer one. It is 7.5 cm. long, 0.2 cm. thick, and 4.8 cm. in outside diameter. A swinging door with a marginal hole, 0.9 cm. in diameter, for the tail, is fixed around a pin. A shallow groove is cut longitudinally on the outer surface of the inner cylinder to slide over the tip of screw A. If the rat is heavy, the diaphragm B is swung out around the pin to allow more space. This has the same number of perforations as the end plate. The entire holder is mounted on a solid, short rod, 6.3 cm. long and 1.25 cm. in diameter, and is fitted into a hollow tube rooted in the center of an iron stand, 15.4 cm. by 12.9 cm. by 2.5 cm. (height). It can be lowered or raised by means of screw C.

\*From the Lilly Research Laboratories and Indiana University School of Medicine, Indianapolis.

Demonstrated at the Meeting of the Federation of American Societies for Experimental Biology, Baltimore, Md., March 31 to April 2, 1938.

Received for publication, June 23, 1938.

†Those workers who do not have shop facilities may communicate with Mr. Elmer Lee, a machinist, 5040 West Washington Street, Indianapolis, concerning the supply of these holders.

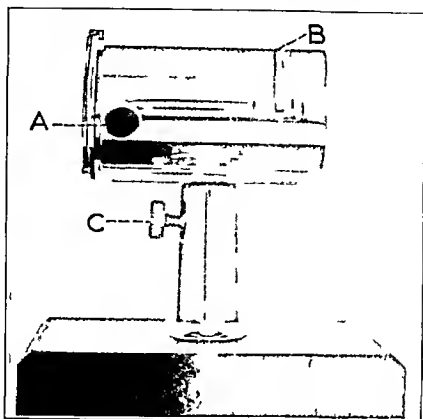


Fig. 1—A rat holder

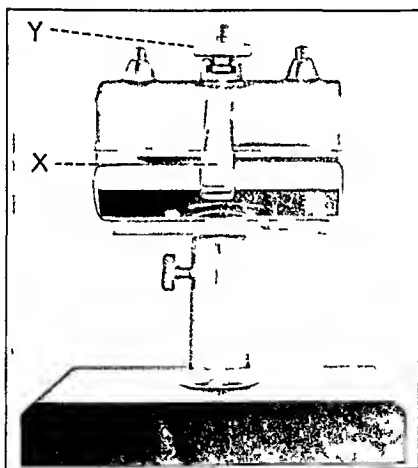


Fig. 2—A guinea pig holder

To prepare for an intravenous injection the gate is left open, the inner cylinder is pulled out four fifths or two thirds of its length, and the rat is allowed to walk into the chamber. After the gate is closed leaving the tail outside, the inner cylinder is pushed as far as possible, without overcrowding the animal, and is fastened by screw A.

The mouse holder is similar to that for the rat, only much smaller. The outer cylinder is 4.1 em. long, 0.2 em. thick, and 3 em. in outer diameter. The end plate has 16 holes, each measuring 0.32 em. in diameter. The inner cylinder is 4.1 em. long, 0.2 em. thick, and 2.7 em. in diameter. The marginal hole for the tail is 0.38 em. in diameter. No diaphragm is needed on the outer cylinder because the holder can easily accommodate mice of all sizes.

The cutaneous veins of the four legs of guinea pigs offer good routes for injections, the forelegs being more preferable than the hind limbs, and the left foreleg more preferable than the right for a right-handed individual. The holder as shown in Fig. 2 will support the body of the animal with the head and forelegs exposed at one end, and the hindlegs at the other. This bivalvular device, 10.4 em. long, opens around the hinges, and locks itself by a spring catch *X* when the upper half is approximated with the lower half. A thin strip of steel sheet is soldered below the lower border so that the upper border can overlap it in closing. This prevents catching of hair or skin between the two halves. The lower half is also provided with semilunar borders at both ends. In order to hold the pig tightly, a concave plate is hung on the inside of the upper half and can be lowered or raised by screw *Y*.

The advantages of these holders are obvious: simplicity of manipulation, complete immobilization of animals, speeding up of the work, capability of the operator to work alone, and ease in cleaning. It cannot be overemphasized that the toxicity data obtained by the intravenous injection in small warm-blooded animals are far more valuable than those by subcutaneous injection in amphibians, such as the frog. Incidentally, the same holders can also serve to secure blood samples, either for cell counting or for microchemical analyses. The exposure of guinea pigs' heads permits the measurement of their pupils, teeth, and so forth. The sizes of these holders are entirely arbitrary to suit our own needs. The mouse holder will accommodate animals weighing between 14 and 35 gm.; the rat holder, between 80 and 200 gm.; and the guinea pig holder, between 250 and 350 gm. The material employed for making our apparatus consists of cast iron and stainless steel. The finished product is all chromium plated, except the base which is painted black. There is no reason, however, why other metals or hard rubber cannot be tried.

#### SUMMARY

Holders for mice, rats, and guinea pigs for intravenous injection have been described.

## A SIMPLE METHOD FOR VISUALIZING PRESSURE AND VOLUME CHANGES APPLICABLE TO OBSERVATIONS ON THE RHYTHMIC VARIATIONS IN THE PERIPHERAL CIRCULATION\*

C WILSLER SCULL, PH D ABINGTON PA

INCIDENTAL to a survey of the methods used in the study of circulation among chronic arthritides<sup>1</sup> it occurred to me that it might be possible to employ a simple device utilizing the elastic properties of thin glass plates, together with the phenomenon of Newton's rings in detecting the changes of volume and pressure associated with the peripheral flow of blood. As far as I am aware, no method employing this principle has hitherto been described.

To test the practicability of such a procedure, an ordinary 'noncorrosive' microscope slide (25 by 75 by 1 mm) and a No. 1 cover glass (22 by 40 mm) were cleaned with chromic acid solution rinsed with water, dried, and then flamed to remove a film of moisture. This preparation simply served to provide fairly clean and relatively nonsticking surfaces. The two glasses were pressed together with a shearing motion. One edge of the cover glass was then attached to the slide near the midline by means of cement or a narrow strip of adhesive tape or of gummed paper. When such a slide is held in a rigidly fixed position and pressure or a weight is applied to the cover glass, a pattern of light and dark bands appears. This optical phenomenon, due to the interference of light waves associated with reflecting surfaces separated by a thin layer of air of nonuniform thickness, is known as Newton's rings. When viewed from the angle of reflection in reflected polychromatic light, the pattern appears as a series of brilliantly colored bands against a white background. When viewed in monochromatic light, the bands are made more clearly evident and appear as dark lines against a lighter background of the color used. The latter arrangement is preferable for observations on peripheral pulsation inasmuch as bright reflected light obscures the pale colored bands which first appear under minimal pressure.

These phenomena have been used in static measurements of the curvature of lenses, and in the determination of the optical regularity of plane surfaces. The proposed use involves the additional utilization of dynamic phenomena which take advantage of the fact that when the pressure applied to the thin deformable glass slip is varied the thickness of the air layer is thereby modified, and this in turn alters the interference pattern. When changes of pressure are exerted in a regular sequence, the impression of a continuous movement of bands is produced which parallels the modification of applied pressure.

\*From the Laboratory of Rheumatoid Diseases Abington Memorial Hospital  
Received for publication June 25 1938



Thus, if such an arrangement of glass plates held in one hand is placed over the wrist with one end of the slide resting on the styloid process of the radius with the cover slip directed downward over the radial artery while slight, but constant, pressure is maintained from the opposite end of the slide, pulsations are made clearly evident by a rhythmic shifting of the colored bands. By fixing the attention on one band or one central circular area, it is possible to recognize a rapid extension of a band from its base line or an enlargement of the circle succeeded by a decrease in diameter, and this in turn is followed shortly by a slight rebound which is succeeded by a return to the original position. The phases of the pulse are thus made visually evident by the momentary "peaks" in the alternating ebb and flow of the bands from their basal levels.

The circulatory pulsations in the fingers may be made evident in a similar fashion. In order to maintain the finger in a fixed position and to apply constant pressure on the slide it is necessary to provide a stage for supporting the finger and a holder for the slide. A simple, but adequate, rack for this purpose has been made with a ring stand, clamps, and a burette holder, arranged so that the finger may be rested upon a block and the slide, held in a burette holder, may be turned downward to bring the slide to a position in which the lower surface of the cover slip touches the finger. If a stand is not available, a substitute may be made by laying the slide on a finger and loosely attaching it by means of strips of adhesive tape to a splint on the opposite side of the finger. The optimal position is that in which the minimal degree of pressure required for producing bands is exerted. Under these conditions pulsations become clearly evident. Obstruction of blood flow to the hand by the application of pressure on the brachial artery results in a prompt cessation of the "movement" of the bands.

Fig. 1 shows the general appearance of the interference bands produced by application of the slide to the finger. Illumination, daylight; exposure, one-tenth second; size, one-half actual.

Fig. 2 illustrates the general sequence of changes which can be recognized readily with the simple device applied over the radial artery or to the finger of a normal subject. It is evident from this diagram that a marked amplification of the actual vertical movement, with respect to the plane of the glass surface imparted to the thin glass, is achieved by following the horizontal movement of the interference band. If it can be assumed that the perceptible change of color, representing the modification in thickness of the layer of air, is indicated by the respective wave lengths of light, it would follow that a movement imparted to the thin glass in the order of 0.0001 mm. is recognizable. Since with such a color change there is a shift of the boundary band equivalent to 5.0 mm., it may be deduced that an apparent amplification factor approaching 50,000 times the actual movement may be realized.

With ordinary cover slips the pattern produced by the lines in the colored field is not uniform, due to irregularities in the surface of the glass. With cover slips, such as are used for blood counting chambers, the bands appear

in a more nearly uniform arrangement. The latter combination while presenting wide bands and a clear cut regular pattern is not essential for recognizing changes in the magnitude of applied pressure. In fact the thicker glass is less sensitive as an indicator of rapid changes and shows a definite lag in following the alternations of applied pressure. In addition, the forces required to modify the pattern are greater than with thinner glass because of the lesser degree of deformability of the thicker glass.



Fig 1—Photograph of sphygmoscope showing interference bands

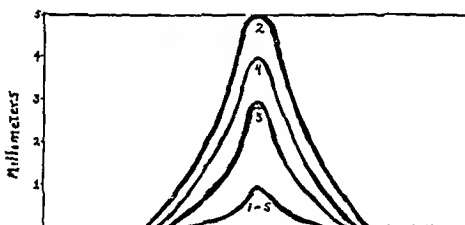


Fig 2—Diagram showing the relative position of an interference band at intervals during a single pulse cycle

Position	Time Seconds Approximate
1	0.0
2	0.2
3	0.3
4	0.4
5	0.5 to 1.0

In order to increase the sensitivity to the rapid alternation in pressure and provide a fairly reproducible pattern a segment of a No. 1 cover slip, cut to 8 mm by 22 mm, was attached to a slide permitting space at each end for clamps. It has also been noted that slight elevation of one or both ends of the cover slip by means of a narrow thin strip of glass or thin copper foil (0.002 inch thick) makes the arrangement more rapidly responsive to changes in applied pressure. This is due to the fact that with the release of pressure from the center of the thin glass there is a slight but definite tendency for the glass to spring back beyond the limit necessary for the appearance of interference bands.

There are several obvious ways in which such a device may be utilized clinically. It might be employed for the purposes served by other forms of oscillometric and sphygmoscopic devices. Among these may be mentioned the detection of the presence or absence of pulsations in a member suspected of having an obstruction, as an oscillometer. In addition to this use, the device may be employed as a sphygmoscope or an indicator for determining the pres-

ence of conditions associated with abnormalities of the pulse pattern, e.g., in showing the relative position of the dicrotic wave in aortic regurgitation and other cardiac states.

To survey further the practicability of the procedure, a preliminary series of observations upon a few normal individuals and several patients with clear-cut cardiac and circulatory diseases have been made. Differences in the pulse pattern have been made qualitatively evident. In cases with aortic regurgitation the dicrotic notch has been recognized as being closer to the base line than in normal subjects. In patients with impaired circulation in the hands and feet absence or decrease of pulsations has been made evident. These observations indicate that the principle may be utilized as a supplement to other methods for the ready demonstration of variations in the peripheral circulation.

The procedure is now presented merely as an accessory method for visualizing the state of circulation at the periphery. Pulsations which are made evident at peripheral points are obviously the resultant of those forces driving the blood through these parts and the elasticity of the tissues. As described, the procedure apparently provides an index of the sudden rhythmic volume changes in the peripheral tissues chiefly determined by the cardiac cycle, and represents roughly the pulse volume changes.

One evident shortcoming inherent in the simplicity of this procedure is that it is not strictly quantitative and includes no method for providing a permanent record of the data for comparative purposes. Refinement with respect to these factors by employing optically plane, thin glass slips, a mechanical stage suitable for adapting the glass at a constant and reproducible pressure, and elaboration with photokymographic accessories for recording the ebb and flow of the interference bands, would meet these difficulties and make possible a better analysis of the pulsations. However, the simple procedure described provides a new and qualitatively adequate means for visualizing the presence of pulsations, together with some of the characteristics of the rhythmic pressure and volume changes in the peripheral tissues.

#### REFERENCE

1. Pemberton, R., and Scull, C. W.: *The Circulation in Chronic Arthritis*, 107, 127 of *Chronic Rheumatic Diseases*, London, 1938, Oxford Medical Publication. Oxford University Press.

## UNRELIABILITY OF OXALATED BLOOD IN DETERMINATION OF ERYTHROCYTE SEDIMENTATION RATE\*

SAMUEL BERG, M D, NEWARK, N J

SINCE its rediscovery by Fahraeus in 1918<sup>1</sup> the suspension stability of plasma, better known as the sedimentation reaction of erythrocytes (designated S R), has been the subject of numerous laboratory and clinical studies aiming to determine its value as a diagnostic and prognostic procedure. The recorded findings of various workers are so confusing and contradictory that the relationship of the sedimentation rate to the condition of the patient is still unsettled. It was simple enough, in the days of limited medical knowledge to draw fundamental conclusions from the manner in which large quantities of drawn blood settled into layers, but modern medicine insists on a more detailed study of the falling rate and its relationship to morbid processes. Various techniques have been suggested, all depending on the use of chemical anticoagulants. This is merely one of a number of maneuvers likely to introduce errors in the final determination, errors which internists, although cautioned about, have not fully appreciated especially as regards the anticoagulant. Too often variations due to technical factors have been assumed to be due to disease processes and undoubtedly this is partly responsible for the contradictory opinions concerning the test. All anticoagulants introduce some degree of error, but in general wider variations have been noted with oxalates than with the others. This is especially important in view of the growing practice of hospital laboratories of using oxalated blood for many routine determinations according to systems devised by hematologists.<sup>2</sup> The tests that are said to give reliable results are the red and white cell counts, platelet and reticulocyte counts, hemoglobin, chemical contents, and sedimentation rate if determined within three hours, and also the erythrocyte volume index and the sedimentation rate if the blood is mixed in the proportion of 1 c.c. per 2 mg. potassium oxalate. The adoption of a system of this kind saves considerable time and trouble of patient and intern, inasmuch as a single bleeding gives the laboratory opportunity to complete the routine determinations now expected of all new admissions to hospitals. This is the main reason for the substitution of oxalate for citrate in determining the sedimentation rate. Another is that dry oxalate can be kept indefinitely in a container ready for use, whereas citrate solutions tend to deteriorate and must be kept sterile if drawn into the syringe when obtaining the blood. Furthermore, the method has been recommended as reliable by good authorities. At one time the author included the sedimentation rate test in routine determinations on oxalated blood from tuberculous patients, but found

\*From the Laboratories of Sea View Hospital, Staten Island, N. Y., and the Newark, N. J. City Hospital. Dr. Harrison S. Martland, pathologist. Dr. George Engel assisted in the latter institution.

the results to be so variable that its dependability as an index of the status and progress of lesions was questioned. By exclusion, the anticoagulant was suspected to be the cause of discrepancies, and, in order to check on this, several experiments were performed, the results of which it is felt deserve recording.

Heparin and hirudin are considered to be the best anticoagulants in that they have very little or no influence on the sedimentation rate, but their expense and uncertainty preclude their routine use. Fluorides may be dismissed from consideration. Up to recent years the most popular method was that of Westergren,<sup>3</sup> in which blood is mixed with citrate solution. As for oxalates, some internists drew attention to undependable results following their use; others published data showing quite consistent readings when the tests were run in duplicate with citrated or heparinized blood. That the question of the influence of various anticoagulants on the sedimentation rate is in need of further study is quite evident from the contradictory conclusions arrived at by various workers, several of whom will be quoted here.

Westergren<sup>4</sup> found that the sedimentation rate of blood citrated according to his technique is not materially affected, but that it is slowed after twenty-four hours. Peterman and Seeger<sup>5</sup> noted variations among different anticoagulants which were difficult to interpret. Pinner, Knowlton, and Kelly<sup>6</sup> ran a series of duplicate tests using citrated and oxalated blood according to Westergren's method (height of column), and found that low sedimentation rates are usually higher in oxalated blood than in citrated blood, and that with high rates the relation is frequently reversed. Ernestene<sup>7</sup> claims that dilution with sodium citrate solution makes accurate readings impossible. Rourke and Plass<sup>8</sup> found that sodium fluoride, potassium oxalate, and potassium citrate retard the falling rate; that the rate of sedimentation varies inversely with the concentration of salt necessary to prevent coagulation; and that increasing the concentration of any of the above anticoagulants always slows the rate. Contrariwise, Wintrobe and Landsberg<sup>9</sup> claim that potassium oxalate in the proportion of 2 mg. per 1 c.c. of blood is a satisfactory anticoagulant and does not depress the rate of settling. They remark that the disagreement between their observations and those of Rourke and Plass can probably be accounted for in part by the somewhat lower concentration of oxalate they used, and then add that they failed to note a consistently depressing effect on sedimentation even with greater quantities of oxalate such as these investigators used. Hinton<sup>10</sup> claims that citrate and oxalate slow the rate by altering the shape and surfaces of the red blood cells and so diminish the agglutinability of the cells. Sasano, Ordway, and Medlar<sup>11</sup> found that oxalates in minimal quantities are very satisfactory in sedimentation rate determinations, yet Table VI in their article shows the average rate of ten cases run in duplicate to be 11.2 mm. by the citrate method and 17.3 mm. by the oxalate method, results which may be satisfactory in that they both are slow but certainly not accurate for comparative purposes. Dorfman and Brooks<sup>12</sup> obtained results which show: first, delay in performing the test causes slowing of the sedimentation rate with both oxalated and heparinized blood; second, the effects of delay are shown much earlier with oxalated blood than with heparinized blood; and third, heparinized blood settles more slowly than oxalated blood. Bannick,

Gregg, and Guernsey<sup>13</sup> express satisfaction with citrate used according to Westergren's method and with oxalate in the proportion already mentioned. Ham,<sup>14</sup> however, thinks that solutions of sodium citrate cause significant slowing of the sedimentation rate due to dilution and to salt effect, and that potassium oxalate in a concentration of 2 mg per 1 cc of blood has no significant effect. Haskins, Trotman, Osgood, and Mathieu<sup>15</sup> state that comparative studies of oxalated blood and blood diluted with citrate showed that the use of oxalate did not introduce clinical error, the test is to be started within three hours of drawing the blood. Mattice<sup>16</sup> found dry oxalate superior to citrate solutions but does not concur with Haskins and Wintrobe in the belief that the test may be set up within three hours of taking the blood, it must be done immediately. At room temperature pathologic blood rapidly changes its sedimentation rate in the direction of normal. Whitby and Britton<sup>17</sup> believe the test to be so sensitive that an exact technique is essential. It is important to set up the blood at the earliest possible moment, results after an hour or two delay are never so reliable as those obtained immediately.

Obviously, such divergent opinions must mean that the test is not as free from intrinsic faults as its technical simplicity suggests. Presumably, certain very important precautions were observed by all those whose opinions were quoted, such as the use of clean glassware and the maintenance of tubes in a perfectly vertical position during the test. Some of the articles did not mention the length of blood column or time intervening between obtaining the blood and performing the test. Because all these are potential sources of error, and because comparison of results obtained by different investigators is unfair unless a uniform technique is used, the procedure followed by us will be given in some detail.

Blood was withdrawn from a vein in the cubital fossa with a dry syringe before breakfast. Citrated specimens were prepared by mixing 4 parts of blood with 1 part of 3.8 per cent sodium citrate solution already accurately measured out in a container. Oxalated specimens were prepared by mixing blood with dry potassium oxalate in the proportion of 1 cc to 2 mg, in practice, 8 cc of blood were transferred to a container in which 0.8 cc of 2 per cent potassium oxalate solution had been dried in a hot air oven. The specimens were then drawn up to the 200 mm mark in tubes having a bore of about 2.5 to 3 mm, and the tubes were set up in a rack in a perfectly vertical position. The tests were performed at room temperature within a half hour of obtaining the specimens. The tubes were cleaned with tap water, alcohol, and ether, and dried with a vacuum pump. The column of clear plasma was read at the end of fifteen, thirty, forty five, and sixty minutes, but *only the final readings are recorded in the tables*. The tubes were read in succession at intervals of twenty seconds, the approximate time taken to set up each one in order. This precaution must be observed for exact results when performing the test simultaneously with many tubes, especially when the rates are fast. Patients are designated alphabetically, the letters representing different patients in each experiment.

*To Test the Consistency of S R With Citrated Blood*—Sufficient blood was withdrawn from each patient to set up 4 columns of blood simultaneously, and

the rates were determined at once. It will be noted from Table I that the fall is quite consistent. This serves as a check not only on the consistency of the sedimentation rate of citrated blood but also on the accuracy of our technique.

TABLE I

CASE	TUBES			
	1	2	3	4
A	19	19	19	17
B	22	24	27	26
C	39	34	37	42
D	73	71	65	70
E	55	55	54	53
F	7	6	8	7
G	42	40	41	42
H	14	14	14	13

*To Note the Consistency of S. R. With Oxalated Blood.*—The above experiment was repeated, using oxalated blood. Table II shows that the fall is quite consistent if the rates are determined without delay.

TABLE II

CASE	TUBES			
	1	2	3	4
A	20	20	18	19
B	32	32	31	30
C	46	43	44	45
D	8	8	7	7
E	105	104	101	103
F	129	127	130	128
G	3	3	3	3
H	18	17	19	18

*To Note Effect of Time Interval on Consistency of S. R. With Oxalated Blood.*—Sufficient blood was drawn and citrated in the manner described to determine the sedimentation rate immediately, and also one, two, and three hours later. Table III shows that the rates are consistent even after an interval of three hours.

TABLE III

CASE	STAT	1 HR.	2 HR.	3 HR.
A	112	105	105	108
B	110	108	109	101
C	35	35	31	32
D	20	24	14	17
E	43	49	43	40
F	6	3	5	4

*To Note Effect of Time Interval on Consistency of S. R. With Oxalated Blood.*—The above experiment was repeated, using oxalated blood. Table IV shows that the greater the time interval between oxalating the blood and determining the sedimentation rate, the greater and more frequent is the retardation in sedimentation.

TABLE IV

CASE	STAT	1 HR.	2 HR.	3 HR.
A	74	78	38	46
B	68	29	15	6
C	17	7	5	2
D	25	15	8	4
E	130	120	110	105

*To Note Effect of Time Interval on S R, Using Citrated and Oxalated Blood in Duplicate Tests*—Blood was drawn from 19 patients and each sample was divided into two portions, one was citrated and the other was oxalated as described. The sedimentation rates were determined on all specimens soon after withdrawal. In 5 cases the rates were determined again two hours later, in 5 other cases, three hours later, and in 9 cases four hours later. The results are shown in Table V.

TABLE V

CASE	SOON AFTER BLEEDING		2 HR. LATER	
	CITRATED	OXALATED	CITRATED	OXALATED
A	80	88	89	63
B	63	53	57	12
C	60	32	75	24
D	16	36	16	11
E	78	100	78	77
			2 hr. later	
F	60	78	65	7
G	73	39	32	5
H	53	48	52	9
I	92	89	95	6
J	99	113	122	7
			4 hr. later	
K	34	36	32	70
L	41	43	37	11
M	44	42	43	45
N	16	18	12	7
O	42	47	34	9
P	120	125	97	16
Q	30	32	28	31
R	56	59	52	22
S	9	11	4	2

On comparing the figures in columns 1 and 2 (citrated and oxalated soon after withdrawal of blood), one will note that there is a rather marked discrepancy in cases C and D, in one case the oxalated specimen showing a rate twice that of the citrated specimen, and in the other case just the reverse. In all other cases the differences, though moderate and not of great practical significance, are still too large for comparative purposes. When tested immediately, a citrated blood will usually show a faster rate than the same blood oxalated. Occasionally the reverse occurs. After an hour or more, the rates are markedly retarded with oxalated blood.

The figures in columns 1 and 3 (citrated blood tested soon after bleeding, and two, three, and four hours later), bear out what was seen in Table I that citrated specimens give readings that are quite consistent over an interval of three hours, and even of four hours.

Comparison of the figures in columns 1 and 4 (citrated blood soon after bleeding, and oxalated blood two, three, and four hours later), shows that these intervals of time give sedimentation rate readings which are ridiculously retarded in 11 out of 19 cases, and markedly increased in one case. It is quite apparent that any material interval of time between oxalating the blood and determining the sedimentation rate makes this procedure very untrustworthy.



*To Note the Effect on the S. R. of Adding Oxalate to Citrated Blood.*—Blood was withdrawn from 9 patients and citrated in the usual manner. Each specimen was then divided into three portions of 3 c.c. each. One portion was left as such; to the second portion was added 1 drop of a saturated solution of sodium citrate; to the third portion was added 1 drop of a saturated solution of potassium oxalate. All three specimens from each patient were then set up and the sedimentation rate determined simultaneously. It will be noted from Table VI that the increased concentration of citrate causes a decrease in the sedimentation rate rather consistently, as expected. When oxalate is added, however, the changes are inconsistent, the rates being much faster in 1 case, much slower in 4 cases, and approximately unchanged in 4 cases. It is evident that oxalate introduces a variable that makes it absolutely unreliable as an anticoagulant in determining the sedimentation rate. Of course, in actual practice much smaller quantities of oxalate are used in performing the test; but, as was noted in Table V, even then the results are undependable soon after mixing, much more so with increasing intervals of time.

TABLE VI

CASE	ORIGINAL	CITRATED	OXALATED
A	27	24	.62
B	113	86	12
C	31	26	8
D	22	20	25
E	36	34	38
F	45	40	18
G	12	8	5
H	3	2	1
I	32	26	6

*To Note Whether Diluted Oxalated Blood Reduces the Error in the S. R.*—It has been shown that blood diluted up to 20 per cent of its volume with 3.8 per cent sodium citrate solution has a sedimentation rate not much different from the same specimen prepared with hirudin or heparin; the resulting increase in rate, if any, certainly does not approach that expected according to results obtained in experiments in which the volume index of blood was decreased by dilution with its own plasma.<sup>10</sup> The question arose as to whether dilution had any tendency to correct the error introduced by an anticoagulant. Accordingly, the following experiment was performed. Blood was citrated with citrate solution by Westergren's technique, and another portion of the same blood was mixed with dry potassium oxalate. The latter was divided into two portions, one of which was then diluted to one-fifth of its volume with oxalated saline (0.2 gm. potassium oxalate per 100 c.c. normal saline). The use of pure normal saline dilutes the anticoagulant effect of the original oxalate, hence the necessity for additional oxalate in the diluent to prevent clotting. The three specimens were set up at once and the sedimentation rate determined.

From the results in Table VII, it will be seen that the sedimentation rates of the diluted oxalated blood (termed wet oxalate) agree more closely with those of citrated blood than do the dry oxalate specimens. Hence, if it is desired to do routine sedimentation rate determinations on specimens of blood prepared with

dry oxalate in the routine way, less aberrant results may be expected if the portion of blood used for this purpose is first mixed with one fifth its volume of oxalated saline, provided there is little delay in the procedure

TABLE VII

CASE	CITRATED	DPY	
		OXALATED	WET OXALATED
A	6	6	6
B	7	8	6
C	12	20	10
D	21	26	20
E	5	12	9
F	14	25	15
G	85	96	68
H	118	122	128
I	48	62	64
J	62	84	64
K	11	13	11
L	15	26	17
M	101	122	112
N	116	116	101
O	32	12	26
P	90	120	99
Q	88	117	90
R	66	99	64
S	61	90	61
T	37	56	38
U	74	89	75
V	4	6	4
W	71	92	72
X	105	101	107
Y	51	90	56
Z	80	110	80

## SUMMARY AND CONCLUSIONS

1 Blood citrated according to Westergren's method shows sedimentation rates which maintain consistent rates for at least four hours. This consistency warrants the acceptance of the sedimentation rate with citrated blood as reliable, any deviation from the true rate due to dilution or salt effect is constant, and has already been discounted in the establishment of the normal rates by this method.

2 Blood mixed with dry oxalate in minimal quantities, i.e., 2 mg of the potassium salt per 1 c.c. of blood, will show sedimentation rates which are fairly reliable only if performed with no delay.

3 More accurate sedimentation rates will result with blood thus oxalated for routine studies if the portion used for the test is diluted with one fifth its volume of oxalated normal saline and tested at once.

4 Sedimentation rate determinations made with oxalated blood are absolutely unreliable if the delay between oxalating and testing is an hour or longer; the longer the delay, the greater the incidence and degree of error.

5 In large institutions where it is desirable to include the sedimentation rate in the routine blood determinations and heavy laboratory schedules make immediate examination impractical, more reliable and accurate results will be obtained by distributing tubes containing 0.5 c.c. of 3.8 per cent sodium citrate solution and having 2 c.c. of blood syringed into this container at the time blood

is drawn for other determinations. This amount (2.5 c.c.) suffices for use in a Westergren tube (200 mm. by 2.5 mm. column). The test may be performed at any time within four hours of citration.

## REFERENCES

1. Fahraeus, R.: Agglutination in Blood, *Hygeia* 80: 369, 1918.
2. Osgood, E. E., Haskins, H. D., and Trotman, F. E.: A Uniform System of Hematologic Methods for Use With Oxalated Blood, *J. LAB. & CLIN. MED.* 16: 476, 1931.
3. Westergren, A.: Studies of the Suspension Stability of the Blood in Pulmonary Tuberculosis, *Acta med. Scandinav.* 54: 247, 1921.
4. Westergren, A.: Sedimentation Reaction in Tuberculosis, *Ergebn. d. inn. Med. u. Kinderh.* 26: 577, 1924.
5. Peterman, M. G., and Seeger, S. J.: Sedimentation Reaction in Children, *Am. J. Dis. Child.* 37: 693, 1929.
6. Pinner, M., Knowlten, K., and Kelly, R.: Sedimentation Reaction of Erythrocytes in Tuberculosis, *Arch. Path.* 5: 810, 1928.
7. Ernste, A. C.: Erythrocyte Sedimentation as Index of Rheumatic Infection, *Am. J. M. Sc.* 180: 12, 1930.
8. Rourke, M. D., and Plass, E. D.: Investigation of Various Factors Which Affect Sedimentation Reaction of Red Blood Cells, *J. Clin. Investigation* 7: 365, 1929.
9. Wintrobe, M. M., and Landsberg, J. W.: A Standardized Technique for the Blood Sedimentation Test, *Am. J. M. Sc.* 189: 102, 1935.
10. Hinton, A. C. R.: The Corrected Erythrocyte Sedimentation Test, *J. LAB. & CLIN. MED.* 18: 711, 1933.
11. Sasano, E. T., Ordway, W. H., and Medlar, E. M.: Factors Which Influence the Sedimentation Rate With Especial Emphasis Upon Effects of Temperature, *Am. J. Clin. Path.* 6: 432, 1936.
12. Dorfman, R. I., and Brooks, C.: The Accuracy of a New Technique for Measurement of the Red Blood Cell Sedimentation, *J. LAB. & CLIN. MED.* 22: 510, 1937.
13. Bannick, E. G., Gregg, R. O., and Guernsey, C. M.: The Erythrocyte Sedimentation Reaction, *J. A. M. A.* 109: 1257, 1937.
14. Ham, T. H.: Seventh Annual Meeting of the American Academy of Pediatrics, *J. Pediat.* 12: 675, 1938.
15. Haskins, H. D., Trotman, F. E., Osgood, E. E., and Mathieu, A.: A Rapid Method for Determination of the Sedimentation Rate of the Red Cells With Results in Health and Disease, *J. LAB. & CLIN. MED.* 16: 487, 1931.
16. Mattice, M. R.: Chemical Procedures for Clinical Laboratories, Philadelphia, 1936, Lea & Febiger, pages 25 and 32.
17. Whitby, L. E. H., and Britton, C. J. Z.: Disorders of the Blood, Philadelphia, 1937, P. Blakiston Son & Co.

156 ROSEVILLE AVENUE

## LABORATORY METHODS FOR TESTING FUNGICIDES USED IN THE TREATMENT OF EPIDERMOPHYTOSIS\*

EILA M BURLINGAME AND GEORGE F REDDISH St Louis, Mo

THE disease condition recognized as epidermophytosis commonly known as "athlete's foot," has increased considerably during recent years. This skin infection now affects approximately one third of the population and is widely and generally distributed throughout most parts of the world. With the increased prevalence of this disease and more general knowledge as to its cause there has been a consequent increase in the use of certain fungicides in treating this condition. The fungicides employed have been selected after clinical experiences have demonstrated their merit for this purpose. In vitro tests have also been made to some extent in which various laboratory procedures have been employed. So far, however, none of the laboratory methods suggested have proved entirely satisfactory.

Probably the first laboratory method of merit was the one proposed by Schamberg and Kolmer<sup>1</sup> in 1922. In this method three of the most common pathogenic fungi were used as test organisms for determining both fungistatic and fungicidal activity. For determining fungistatic activity the test organisms were inoculated on Sabouraud's agar slants containing various concentrations of the compounds tested and then observed for inhibition of growth. In the fungicidal test the same test organisms were grown on Sabouraud's agar, suspended in saline solution, and mixed with different concentrations of the fungicides tested, transfers being made to slants of Sabouraud's agar at time intervals from fifteen minutes to twenty-four hours.

This method, or some modification of it, has been used by many other workers in this field and is still quite generally employed. Myers and Thienes<sup>2</sup> used essentially the same method in reporting on the fungicidal activity of certain volatile oils. Kingery and Adkisson,<sup>3</sup> Woodward, Kingery, and Williams,<sup>4</sup> and Kingery, Williams, and Woodward,<sup>5</sup> used certain modifications of the method of Schamberg and Kolmer in their extensive studies on volatile oils, stearoptens, and phenol derivatives. Klarmann, Shternov, and Gates<sup>7, 8</sup> made use of essentially the same technique in their studies on the fungicidal activity of derivatives of parachlorophenol and orthochlorophenol. Stovall, Pessin, and Almon,<sup>9</sup> and Maplestone and Dey<sup>10</sup> used modifications of this test in studies on thymol and certain other fungicidal substances. McCrea<sup>11</sup> in 1931 suggested that the Schamberg and Kolmer method, or a slight modification of it, be adopted as the standard method for testing fungicides.

\*From the Bacteriology Laboratory of the Lambert Pharmacal Company, St Louis.  
Received for publication July 20 1938.

While this method of test has served a useful purpose in separating those substances which are fungicidal and may be effective for treating epidermophytosis from those which may not have any merit for this purpose, there are, however, certain objections to this method which should be mentioned at this time, especially since this procedure has been suggested as a standard test. The first objection to this test is that it does not in any way simulate practical conditions of use. In fact, the conditions created in this test are just opposite to those met with in practice. Suspending the organisms and spores in saline and shaking with glass beads to break up the clumps and matted mycelium before exposing to the action of the fungicide is quite different from the condition of the organisms as they occur in infected skin. Under the conditions of actual infection the organisms are closely matted and embedded in tissue. Testing the killing power of fungicides after breaking up the matted mycelium and spores of the test organisms is not logical and does not simulate practical conditions in the least. Also, no clinical evidence has been offered to prove that this arbitrary *in vitro* test is a satisfactory means of testing such fungicides. Since the method is an arbitrary laboratory test, there should be supporting clinical tests to show that fungicides passing this test will be effective in practical use and that those not passing it will be ineffective in clinical practice.

Another serious objection to the method of Schamberg and Kolmer as described by them is that in transferring from the mixture of fungicide and culture no precautions are taken to prevent inhibition of growth on the subculture slants. The method specifies that several loopfuls of the fungicide-culture mixture are transferred directly to slants of Sabouraud's agar. Since enough of the fungicide may be and probably is carried over to the medium to exert a fungistatic effect, false readings are likely to be made, and inhibition of growth may be interpreted as actual killing. These defects in this method are such that the test in its present form should not be used for testing fungicides which are intended for use in treating epidermophytosis.

Another method for testing fungicides that has been used by certain workers is the phenol coefficient test. Emmons<sup>12</sup> was among the first to use this technique in testing the fungicidal activity of some common disinfectants. The highest dilutions of disinfectants which killed the fungi used as test organisms in five minutes and thirty minutes were divided by the highest dilutions of phenol accomplishing the same result, giving a figure which was designated as the phenol coefficient. Similar technique was employed by Gomez-Vega,<sup>13</sup> Hasseltine and Hopkins,<sup>14</sup> and Dunn.<sup>15</sup>

The phenol coefficient test does not lend itself well to the testing of fungicidal activity against fungi other than bacteria. The method is designed for the use of bacteria as test organisms and is not suited to the use of those fungi which cause epidermophytosis. The medium employed is not suitable for normal growth of these fungi; it is difficult to secure uniform distribution of the organisms in broth; the amount of fungicide-culture mixture transferred is too small; the test is not sufficiently severe and comparison with phenol is not necessary nor even desirable. Also, no information is obtained as to the penetrating properties of

the fungicides tested. For these and other obvious reasons the phenol coefficient method is not suitable for testing fungicides.

Other methods of testing fungicides have been suggested from time to time during the past few years. Strickler<sup>16</sup> used collodion sacs containing suspensions of fungi for testing the fungicidal activity of elemental iodine. This method is well suited for the purpose intended but would not be applicable as a method for testing other kinds of fungicides. Sharlit and Muskatbht<sup>17</sup> and Sharlit<sup>18</sup> used what they have designated as the "membrane method" which is best suited for testing volatile substances, such as thymol, etc., and water soluble fungistatic compounds. The fungicide tested is incorporated into collodion which is then spread inside test tubes into which the agar medium is slanted. The test organisms are then inoculated on the surface of the slants and incubated. If the chemical substances are volatile, or water soluble, enough may be absorbed into the medium to inhibit the growth of the fungi used. This is a test for fungistatic activity, however, and is not a test for fungicidal properties and, therefore, should not be used for testing fungicides.

Smyth and Smyth,<sup>19</sup> in their study on the fungicidal activity of pine oil made use of a new method of test which possesses more possibilities than any of those previously suggested. The test organisms fungi causing skin diseases, are grown on Sabouraud's agar and bits of the fungus growth 1 mm square are removed from the agar slant and are used as the test culture. After exposing these small squares of the matted growth to the action of the fungicide for specified time periods, they are removed, rinsed in 95 per cent alcohol to remove the excess fungicide, and then washed in saline to remove the excess alcohol. The squares are then transferred to Sabouraud's agar and incubated at 20° C for fourteen days. The time periods of test range from five to thirty minutes. This method is far more practical than others suggested for this purpose, and in addition eliminates the possibility of fungistatic activity. The time periods of test also are reasonable and logical.

Probably the most objectionable feature of this method is the difficulty of cutting the fungus growth into pieces 1 mm square and then removing the agar from the bits of growth. There is no need for using such small pieces of culture, and also it is unnecessary to remove the agar. Instead of rinsing in 95 per cent alcohol, it is sufficient to simply rinse out the fungicide in water, but using a time period sufficient to remove all of it. This would hardly be accomplished in the ten second time period specified in the method. Furthermore, it is not necessary to incubate cultures of the fungi employed for one to two months before using as test organisms. A much shorter time period is sufficient. Because of the very small amount of culture used in this method, the test is not sufficiently severe to offer the usual margin of safety.

In spite of previous work in this field there still seems to be a need for a suitable laboratory method for testing fungicides. The methods already employed do not simulate practical conditions, do not test for penetration, have not been sufficiently checked by clinical tests, and are not sufficiently severe. For certain other technical reasons the methods proposed in the past do not seem applicable for testing fungicides intended for the treatment of epidermophytosis.

## EXPERIMENTAL

We have recently had occasion to make a laboratory and clinical study of fungicides which are recommended for the treatment of epidermophytosis or "athlete's foot." Since the laboratory methods of tests available did not seem practical enough to indicate whether or not the fungicides tested would be effective under clinical conditions of use, we found it necessary to develop a new severe test which more nearly approaches clinical conditions met with in practice. The test, as finally perfected, was then checked by extensive clinical tests. It was found that those fungicides which pass this test within the minimum time period of the test are effective in the treatment of epidermophytosis and that those which do not pass the test in the maximum time period are not clinically effective.

Fungicides recommended for treating this condition must of necessity kill or inhibit the fungi causing it. In the past most investigators in this field have made use of more than one test organism and usually efforts were made to use those fungi which are actually involved in epidermophytosis. This is not only desirable but necessary, since there is a certain amount of specificity in the fungicidal activity of many preparations used for this purpose. Somewhat typical of the test organisms employed are those used by Gould and Carter,<sup>20</sup> namely, *Epidermophyton interdigitale*, *Epidermophyton purpureum*, and *Epidermophyton gypseum*. These three fungi are the ones most commonly associated with ringworm of the feet and toes, or so-called "athlete's foot."

In our studies five such test organisms were employed. The following fungi were used: *Epidermophyton interdigitale*, *Trichophyton purpureum*, *Trichophyton gypseum*, *Epidermophyton inguinale*, and *Trichophyton rosaceum*. All of these organisms are not present in every case of epidermophytosis and they are not always present in pure culture. They do cause this condition in pure or mixed cultures, and fungicides must kill all of them if they are to constitute an effective general treatment for this condition.

## FUNGICIDAL TEST

The laboratory method for testing for fungicidal activity employed by us simulates practical conditions more closely than those previously described and suggested for this purpose. This method is as follows:

Using the test organisms listed above, each organism is streaked over the entire surface of Sabouraud's agar (20 c.c.) in a 9 cm. Petri dish, using a dry sterile cotton swab inoculated with cultures of these organisms which had been grown on Sabouraud's agar for five days at room temperature. These plates are incubated at room temperature for five days. The agar cultures are then cut into 1 cm. squares, or into disks by means of a cork borer, 1 cm. in diameter. The fungicide to be tested is poured over the surface of the cultures so as to entirely flood the plates, which requires 15 c.c. or more for each plate. After five, fifteen, and thirty minutes, one of the squares or disks of culture and agar from each plate is removed and placed into 10 c.c. of sterile broth. The excess fungicide is washed out of the matted culture by shaking the broth tube lightly for five minutes. At the end of this time

the block of culture is removed from the broth and spread with the culture side down over the surface of a sterile slant of Sabouraud's agar. These slants are then incubated at room temperature for three weeks and observed for growth. An effective fungicide should kill these test organisms within five minutes.

This test is simple, requires very little material, makes use of those organisms which are the most common cause of epidermophytosis, makes use of a solid medium in which these organisms grow most luxuriantly, uses a large inoculum, and avoids satisfactorily any bacteriostatic effect of the fungicides tested. The method is practical, as has been proved by clinical tests, and has been found to be satisfactory for the purpose of indicating which fungicides will be effective under practical conditions of use and which will not.

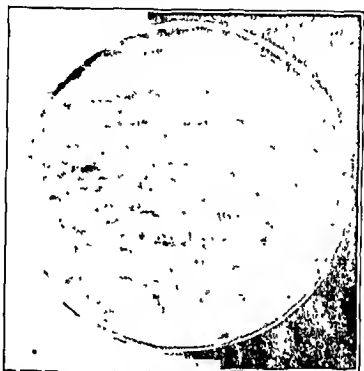


Fig. 1.—Plate showing even distribution of one of the test organisms (*T. interdigitale*) when applied with cotton swab to plate of Sabouraud's agar.

It was found that those preparations which kill these test organisms under the conditions of this test within five minutes are clinically effective in the treatment of this infection. Clinical tests on 30 patients suffering from "athlete's foot" showed that such fungicides are effective in clinical practice. Clinical tests on 30 other patients also showed that a preparation which did not pass this test—that is, did not kill these test organisms—in thirty minutes, was ineffective in the treatment of epidermophytosis.

It is apparent that this laboratory method of test is satisfactory for the purpose of designating which fungicides will and which will not be effective in the treatment of epidermophytosis. It is also evident that this method is more practical, more simple, and that it more nearly simulates conditions as they exist in practice than the other methods which have been suggested. Comparative tests now in progress also indicate that concordant results are readily obtained with this method by different workers. It is for these reasons that the above method is suggested as suitable for testing fungicides recommended for use in the treatment of epidermophytosis.



## FUNGISTATIC TESTS

In the fungistatic tests used in the past the chemical substances tested were simply incorporated into the media used, either dextrose broth or Sabouraud's agar, into which the test organisms were then inoculated, incubated, and observed for inhibition of growth. While this method is helpful in showing which



a.



b.

Fig. 2.—Ointment A on (a) Sabouraud's agar and (b) on Sabouraud's agar containing 10 per cent blood serum, showing the slight difference in zone of penetration and fungistatic activity of this ointment in the presence and absence of blood serum.

substances will inhibit the growth of fungi and which will not, it offers no means of measuring penetration of the fungistatic substance. *Bacteriostatic* activity of antiseptics is tested by means of the serum-agar plate method or the agar cup-

plate method as described by Reddish<sup>21</sup> and U S Department of Agriculture Circular No 198<sup>22</sup> Under these conditions test is made for penetration as well as inhibition of growth Similar technique may be employed in testing for fungistatic activity of various kinds of preparations which are applied for long periods of time

Ointments, salves, powders, etc, which are used in treating epidermophytosis may be tested for fungistatic activity as follows Add 2 c c of sterile normal horse serum to 18 c c of Sabouraud's agar, which has been melted and cooled to 45° C, and pour into 9 cm Petri dishes After the agar has hardened, streak each plate with one of the test fungi, using a dry sterile cotton swab The preparation being tested is then added to the surface of the agar Ointments and salves are melted and streaked on the surface of the inoculated agar by means of a glass rod, and powders are applied to the surface with a small spatula These are applied to but a small portion of the plate, the ointment along a streak about 10 cm wide and extending across the middle of the plate and the powders applied to an area of about 1 cm square The plates are incubated at room temperature for five days If the preparation tested is fungistatic, there will be a clear zone in the medium around the area where it was applied The extent of this clear zone shows the amount of penetration and the effectiveness of the fungistatic ingredient

Liquids and jellies which are applied for long time periods in treating epidermophytosis are tested by the agar cup plate technique<sup>21 23</sup> The serum agar is prepared as for the agar plate method but when it is poured, a vial 10 cm in diameter is placed in the center of the plate and left there until the agar has solidified, after which it is removed and the small cracks and crevices in the resulting cup are filled with two or three drops of melted agar The entire surface of each plate, including the cup, is then inoculated with one of the above test organisms After this has been done, 0.5 c c of the liquid or jelly being tested is placed into the cup, the plates covered with an unglazed clay top, and incubated at room temperature for five days Penetration and fungistatic activity will be shown by a clear zone around the cup

Although we have made no clinical tests with ointments, salves, powders, etc, which show penetration and fungistatic activity by these tests, it can fairly be assumed that such preparations will be beneficial under practical conditions of use In the first place the agar plate and the agar cup plate tests employed are almost identical with the standard Food and Drug Administration methods used for testing for inhibitory power of similar antiseptic preparations, the only essential differences being the use of Sabouraud's agar and the five selected fungi for test organisms The test is severe since it requires activity in the presence of blood serum, penetration through a medium simulating body tissue to some degree, and definite inhibition of the test organisms to a measurable distance from the site of application of the preparations tested For these reasons we consider these tests for fungistatic activity sufficiently practical to be interpreted in terms of effectiveness under clinical conditions of use Those preparations which penetrate this medium and exert fungistatic effect on the test organisms used will be of some effectiveness under clinical conditions of use

## REFERENCES

1. Schamberg, J. F., and Kolmer, J. A.: Studies in the Chemotherapy of Fungus Infections, *Arch. Dermat. & Syph.* 6: 746, 1922.
2. Myers, H. B., and Thienes, C. H.: The Fungicidal Activity of Certain Volatile Oils and Stearoptens, *J. A. M. A.* 84: 1985, 1925.
3. Kingery, L. B., and Adkisson, A.: Certain Volatile Oils and Stearoptens as Fungicides, *Arch. Dermat. & Syph.* 17: 499, 1928.
4. Woodward, G. J., Kingery, L. B., and Williams, R. J.: The Fungicidal Power of Phenol Derivatives. I. Effect of Introducing Alkyl Groups and Halogens, *J. LAB. & CLIN. MED.* 19: 1216, 1933.
5. Woodward, G. J., Kingery, L. B., and Williams, R. J.: The Fungicidal Power of Phenol Derivatives. II. Strength in the Presence of Proteins, *J. LAB. & CLIN. MED.* 20: 950, 1934.
6. Kingery, L. B., Williams, R. J., and Woodward, G.: Further Studies in Fungicides: Comparative Evaluation of Phenol Derivatives by Modified Laboratory Procedure, *Arch. Dermat. & Syph.* 31: 452, 1935.
7. Klarmann, E., Shternov, V. A., and Gates, L. W.: The Bactericidal and Fungicidal Action of Homologous Halogen Phenol Derivatives and Its "Quasi-specific" Character. I. Derivatives of Para-chlorophenol, *J. LAB. & CLIN. MED.* 19: 835, 1934.
8. Klarmann, E., Shternov, V. A., and Gates, L. W.: The Bactericidal and Fungicidal Action of Homologous Halogen Phenol Derivatives and Its "Quasi-specific" Character. II. Derivatives of Ortho-chlorophenol, *J. LAB. & CLIN. MED.* 20: 40, 1934.
9. Stovall, W. D., Pessin, S. B., and Almon, L.: The Effect of Thymol on the Progress of Rabbit Moniliasis, *J. LAB. & CLIN. MED.* 20: 572, 1935.
10. Maplestone, P. A., and Dey, N. C.: Laboratory Tests on the Fungistatic and Fungicidal Effect of Various Substances, *Indian J. M. Research* 25: 603, 1938.
11. McCrea, A.: A Proposed Standard Method for the Evaluation of Fungicides, *J. LAB. & CLIN. MED.* 17: 72, 1931.
12. Emmons, C. W.: Fungicidal Action of Some Common Disinfectants on Two Dermatophytes, *Arch. Dermat. & Syph.* 28: 15, 1933.
13. Gomez-Vega, Paulina: Mycostatic Studies on Certain Moniliae and Related Fungi, *Arch. Dermat. & Syph.* 32: 49, 1935.
14. Hasseltine, H. C., and Hopkins, E. W.: Fungicides. II. In Vitro Tests With a Number of Chemicals on Yeastlike Organisms and Other Fungi, *J. LAB. & CLIN. MED.* 21: 288, 1935.
15. Dunn, C. G.: Fungicidal Properties of Sec-Amyltriethersol, Orthohydroxy-phenylmercuric Chloride and a Mixture of the Two, *J. Infect. Dis.* 61: 31, 1937.
16. Strickler, A.: Elemental Iodine: A New Technique for the Evaluation of Its Fungicidal Properties, *J. LAB. & CLIN. MED.* 18: 49, 1932.
17. Sharlit, H., and Muskatblit, E.: A Search for a New Method for the Determination of the Fungicidal Action of Chemicals, *Arch. Dermat. & Syph.* 25: 615, 1932.
18. Sharlit, H.: The "Membrane Method" for Determining the Fungicidal Action of Chemicals, *Arch. Dermat. & Syph.* 31: 217, 1935.
19. Smyth, H. F., and Smyth, H. F., Jr.: Action of Pine Oil on Some Fungi of the Skin, In Vitro, *Arch. Dermat. & Syph.* 26: 1079, 1932.
20. Gould, A. G., and Carter, E. K.: Fungistasis in Ringworm of the Toes and of the Feet, *Arch. Dermat. & Syph.* 22: 225, 1930.
21. Reddish, G. F.: Methods of Testing Antiseptics, *J. LAB. & CLIN. MED.* 14: 649, 1929.
22. U. S. Department of Agriculture, Circular 198, 1931.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**SULFANILAMIDE**, Experimental Study of Behavior of, Adair, F. L. Hesseltine, H. C., and Hac L. R. J. A. M. A. 111 766 1938

Sulfanilamide has been found in the cervical secretion and menstrual fluid, but in amounts so small that its bactericidal action on the gonococcus is questionable.

The criterion of cure of gonorrhea should be based if possible on cultural studies as well as smears.

Sulfanilamide is excreted in breast milk, both free and as the acetyl derivative. The milk level is considerably above the blood level, and the drug is excreted in the milk for some time after the blood level is negligibly low. With doses of 2 and 4 gm (30 and 60 grains) the total amount excreted was never greater than 15 per cent of the amount of the drug administered. It was still being excreted in small amounts seventy-two hours after medication had been discontinued.

Sulfanilamide is transmitted to the placenta and fetus of the rabbit and is associated with a marked increase in the mortality of the young. Sulfanilamide has also been found in the placenta and cord blood of the human being.

Until more is known of the tolerance of the human fetus and of the newborn for sulfanilamide, the drug should be administered only with the utmost caution during pregnancy and the period of lactation. If administered to the mother breast feeding should be discontinued during the period that sulfanilamide is excreted in the milk.

**PNEUMONIA**, Use of Serum in the Treatment of the Higher Types of, Plummer, N. J. A. M. A. 111 694, 1938

Pneumonia of the higher types is an important part of the pneumonia problem. In a collected series of 6,545 cases of pneumococcal pneumonia, over 50 per cent of the cases were of the higher types, 30 per cent being of types IV, V, VII, VIII, and XIV.

Antipneumococcus serum was used in 111 cases, with a rather marked clinical response and an appreciable effect on the mortality rate for the combined series of cases of pneumonia of types IV, V, VII, VIII, and XIV. At present there are available refined and concentrated preparations of horse and of rabbit serum that are high in antibody content and almost entirely free from reaction-causing substances. With such products the prospects are excellent for obtaining increasingly better results in the treatment of all types of pneumococcal pneumonia.

**DIABETES MELLITUS**, Size of Red Blood Corpuscle in, Mohr, C. F. Am. J. M. Sc. 196 67, 1938

Studies of the red blood cell counts, hemoglobin, and hematocrit determinations have been made in 42 cases of uncomplicated diabetes mellitus, 18 cases of diabetic acidosis, and 4 cases of diabetic dwarfism.

The mean corpuscular volume was found to be 95 cmm, or above, in 14.3 per cent of the uncomplicated diabetic and in 35 per cent of the cases of diabetic acidosis. This increase in corpuscular volume of the red blood cells could not be attributed to the acidosis per se.

**ACETONE:** Stable Sodium Nitroprusside Solution for Acetone Bodies in Urine, Ingham, J. Brit. M. J., Aug. 13, p. 348, 1938.

The reagents for the improved test are:

Solution No. 1.—Ammonium sulfate solution:

200 c.c. saturated solution of ammonium sulfate

200 c.c. ammonium hydroxide, sp. gr. 0.88

Solution No. 2.—Sodium nitroprusside solution:

10 gm. sodium nitroprusside crystals dissolved in 90 c.c. distilled water, and

1 c.c. concentrated nitric acid then added.

*The Test:* To 5 c.c. of urine in a test tube, add 5 c.c. of solution No. 1 and mix. Then add 1 c.c. of solution No. 2, shake, and allow to stand for a minute before judging the color reaction. A deep purple color will then have appeared if 0.1 per cent or more of acetone bodies is present. If only a very faint trace is present, the color will be merely deep red.

The saturated solution of ammonium sulfate used in solution No. 1 should be prepared as follows: Place 780 gm. of ammonium sulfate in a 2 liter flask, and add 1 liter of boiling distilled water. Shake immediately until dissolved, then allow to cool to room temperature. Decant the supernatant solution (or filter if necessary).

**CALCULI:** Blood Calcium, Phosphorus and Phosphatase in Urinary Lithiasis, Griffin, M., Osterberg, A. E., and Braasch, W. F. J. A. M. A. 111: 683, 1938.

In cases of urinary lithiasis the values for blood calcium, phosphorus, and phosphatase exhibit no common change which can be termed characteristic of the group. Minor variations in the value for blood phosphorus, particularly a lowered value, are not unusual, but the concentration of blood calcium is very constant.

Patients with a high value for blood calcium or phosphatase who have urinary calculi should undergo a thorough investigation for some other coexistent pathologic condition.

In this study, hyperparathyroidism was found to be an etiologic factor in less than 0.2 per cent of the 1,206 cases of urinary lithiasis.

**PNEUMONIA,** Atypical, An Acute Infection of the Respiratory Tract With, Reimann, H. A. J. A. M. A. 111: 2377, 1938.

In a series of 8 cases of an unusual, uniform, severe infection of the respiratory tract the disease was not caused by the virus of epidemic influenza or psittacosis, nor was it like other commonly described diseases.

The author was, therefore, led to regard it as a separate disease entity, pending the outcome of further experimental studies. The infection occurred in adults and began as a mild infection of the respiratory tract; this was followed by severe, diffuse, atypical pneumonia, and in 2 cases by the symptoms of encephalitis. Dyspnea, cyanosis, hoarseness, cough without sputum, drowsiness, and profuse sweating were the chief characteristics. The disease lasted several weeks. A filtrable infectious agent recovered from the nasopharynx of one patient and from the blood of another may have been etiologically related to the infection, but the evidence is incomplete. Experiments to clarify this point are under way.

**PLACENTA,** Pathology of, Bartholomew, R. A. J. A. M. A. 111: 2276, 1938.

Infarction of the placenta is the most important pathologic condition to which this organ is subject, not only from the standpoint of frequency, but also because of its relationship to toxemia of pregnancy and maternal and fetal mortality.

The predisposing causes of placental infarction are, first, the hypercholesterolemia of pregnancy, which favors the accumulation of lipid cells at points of stress or injury of the placental arteries, similar to that seen with coronary occlusion, and, secondly, the trauma of fetal movements, which not only tends to cause deposits of lipid cells at points of injury to the placental arteries, but also may break the endothelium over such a deposit or actually bring about rupture of the vessel, thus causing rapid thrombosis.

Toxemia of pregnancy probably results from the absorption of poisonous protein split products of placental autolysis

The poisonous products of placental autolysis are probably peptone, histamine, and guanidine. The pathologic effects of these poisons satisfactorily explain the symptoms and pathologic changes of toxemia of pregnancy.

The peculiar eclamptogenic character of placental autolysate is probably explained by the higher content of arginine in placental tissue, from which guanidine may be obtained.

Toxemia of pregnancy is associated with definite types of acute infarction of the placenta. In examining "unknown" placentas it is possible to diagnose pre-eclampsia, eclampsia, or abruptio placentae in 90 per cent of the cases.

#### BLOOD CHEMISTRY, Postmortem Determinations Hamilton R C Arch Path 26 1135, 1928

The concentrations of sugar, nonprotein nitrogen and chlorides in blood obtained post mortem vary greatly, and their determination is of little value.

Creatinine is the only stable chemical constituent of post mortem blood.

The creatinine content of blood obtained post mortem is no indication of the amount of renal damage present.

A post mortem creatinine determination cannot be regarded as an index of renal function during life.

Chemical analysis of blood obtained post mortem is recommended because of its value in a few cases.

#### ANEMIA Familial Hemolytic, Dimensions of Red Cells in Hill, J M J A M A 111 2179, 1938

Microcytosis was found on at least one occasion in 6 of 7 cases of familial hemolytic anemia, while spherocytosis was found in 5 cases.

Spherocytosis may be absent in the presence of complementing factors, such as cirrhosis of the liver.

Microspherocytosis was observed in one patient during crisis.

No significant correlation could be established between spherocytosis and the severity of the disease, onset of crisis, rapidity of regeneration of the red blood cells, or effect of splenectomy.

#### PREGNANCY TEST, The Xenopus (S A Clawed Toad), Elkan E R Brit M J 2 1253, 1938

The xenopus test allows a diagnosis of early pregnancy to be made within less than twenty four hours.

No animals need be killed to obtain the result.

The reliability of the test does not seem to differ from that of the Aschheim Zondek or the Friedman reaction.

The technique of this test is comparatively simple and very suitable for experimental work on the anterior pituitary like hormone of pregnancy.

For the test, 2 cc of untreated urine or 1 cc of extract is injected into the lymph sac under the dorsal skin of as many female toads as one cares to use. Some observers inject into the leg or into the peritoneal cavity. The toads are slippery and difficult to hold, the easiest way to deal with them is to hold them in a coarse meshed net and to inject through the meshes. There is no immediate reaction to ordinary urine. If extract is used which still contains a trace of alcohol or acetone, the toads react by secreting mucus from the skin glands in the neighborhood of the injection. The question of the "toxicity" of the urine seems to arise very rarely.

After the injection has been given, the toads are put into test jars where they sit on perforated platforms, so that they have no chance of eating their own spawn.

During the test the jars are kept at a temperature of 26° C. The shortest time observed between injection and oviposition was four hours and fifty minutes; the longest, twelve hours.

The eggs, little round balls, half black, half white, and of about 1 mm. in diameter, are covered with a sticky gelatinous substance. Normally these animals do not lay their eggs in bulk but distribute them over a wide area, sticking them on to water weeds one by one. In the test jar the eggs either stick to the underside of the platform or fall to the bottom of the jar. Their number varies enormously. Anything from five to six eggs upwards can be counted as a positive reaction. Tests in which only one or two eggs have been laid by one or two toads should be repeated. They will usually be found to be negative. In the absence of males these eggs are, of course, unfertilized. It should be emphasized that spontaneous ovulation in this species does not take place in captivity or under laboratory conditions. Females, even if kept under the best possible conditions, will never ovulate, except in the presence of a male.

#### **TYPHOID FEVER, Agglutination in the Diagnosis of, and the Typhoid Carrier Condition, Bhatnager, S. S. Brit. M. J. 2: 1195, 1938.**

A strain of *Bact. typhosum* which gives rise to pure Vi agglutination has been identified.

With the help of this strain the Vi antibody is shown to be produced in every case of typhoid fever, the inoculated individuals producing a higher Vi titer than the uninoculated.

Vi agglutination is shown to be a more reliable method of diagnosing typhoid fever in the inoculated than the time-honored O and H types of agglutination.

A correlation between the typhoid carrier condition and the presence of Vi antibody in the serum is described. The employment of Vi agglutination in the routine carrier examination and in tracing the source of typhoid infection is suggested.

#### **SULFANILAMIDE, Studies on the Mechanism of the Action of, Lockwood, J. S., Coburn, A. F., and Stokinger, H. E. J. A. M. A. 111: 2259, 1938.**

The effectiveness of sulfanilamide therapy is related to the type of lesion.

The function of the microorganism, which is strikingly depressed by sulfanilamide, is its capacity to invade tissue.

The effect of the drug on bacterial invasiveness seems to be influenced by the amount of debris present in the lesion.

These phenomena are being investigated experimentally to determine the mode of action of sulfanilamide.

#### **TUBERCLE BACILLI, Spontaneous Lysis of, on Artificial Culture Media, Steenken, Wm. Jr. Am. Rev. Tuberc. 38: 777, 1938.**

It has been shown that ripe cultures of human strains of the Rv variants undergo liquefaction on gentian violet egg and plain egg media, giving rise to a secondary, resistant growth of a different colony and bacillary morphology. The microorganisms from the liquefied area did not produce tuberculosis in the guinea pigs as did the original mother colony. Neither would they grow on any of the media mentioned. However, the secondary resistant type colony, although it did not produce progressive tuberculosis, grew on all of the laboratory media mentioned.

It was observed that the resistant colonies produced skin hypersensitivity in guinea pigs when inoculated intraperitoneally, intratesticularly, and subcutaneously, and that this might persist for one year or longer. When the guinea pigs were killed at the end of a year, autopsies showed no macroscopical lesions, except occasionally at the site of

injection of the intratesticularly infected animals, and the lesions were generally accompanied by a small amount of fibrosis without any demonstrable acid fast microorganisms. Early lesions at times were caseous and acid fast bodies of marked pleomorphisms could be demonstrated.

The above lytic phenomenon is not only inherent in the H37 Rv culture, but has been shown to exist with other Rv variants of human origin. Also all of the cultures used in this experiment were of human origin as proved by the present methods of differentiation.

It has been demonstrated that there is a definite lytic principle in the filtrate of the culture which acts upon the living bacilli within a definite range of pH.

It has also been observed that the bacillary morphology of the resistant colony is markedly different from the Rv variant.

#### ANEMIAS Hemolytic, Hemolysins as the Cause of Clinical and Experimental, Dameshek, W D, and Schwartz S O. *Am J M Sc* 196 769 1938

Isohemolysins of the immune body type were discovered in the serum of 3 cases of acute hemolytic anemia.

Anti guinea pig hemolytic serum was prepared by the injection of guinea pig red blood cells into rabbits. This serum possessed all the immunologic properties of the serum found in the clinical cases.

Hemolysis of the red blood cells of the guinea pig *in vivo* followed the injection of this serum.

By varying the dosage of anti guinea pig hemolytic serum various types of hemolytic syndromes were produced: fulminating hemolytic anemia with hemoglobinuria, acute hemolytic anemia and subacute hemolytic anemia.

Various types of blood pictures could be reproduced: microspheroctosis, increased erythrocyte fragility, reticulocytosis, pseudomacrocytic blood picture, and so on.

The spherocyte is a small thin red blood cell unaltered in volume though small in diameter, and unusually fragile to hypotonic salt solutions. Our observations point to the conclusion that spherocytosis is due to the activity of hemolysin and not to an abnormal anatomic peculiarity or to a disturbed formation of cells in the bone marrow. Since increased fragility is a function of the increased thickness of the red blood cell it is dependent upon the same cause.

The authors believe that hemolytic syndromes are due to hemolysins possibly of different types and present in different amounts functioning slowly in some cases and violently in others. The various blood pictures of the hemolytic anemias viz anemia, spherocytosis, increased fragility, and reticulocytosis, are in all probability due to the effects of the varying activity of hemolysins, and modified by the individual's power to react.

Since the experimentally produced hemolytic syndromes and the numerous clinical types are closely comparable, the chief differences in the clinical syndromes may be a matter of the amount of functioning hemolysin present.

#### BLOOD SEDIMENTATION, Microsedimentation in Infants and in Children, Rogatz, J L. *Am J Dis Child* 56 1037, 1938

The Smith micrometric method was compared with the accepted Cutler macrometric method in 100 tests, performed during a period of fourteen months by different workers on 75 infants and older children of varying ages and with different illnesses. On the basis of the results it is concluded that the micrometric method, which requires minimal amounts of capillary blood, obtainable by pricking the finger, is a satisfactory test, is easy to use, and is completely reliable in its accuracy. One need not hesitate to replace existing macrometric methods with this test.



---

Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

---

## Synopsis of Clinical Laboratory Methods\*

THIS is an excellent guide for the graduate clinical technician as well as a text for the student. It covers all phases of clinical laboratory procedure, presenting an unusual wealth of material and choice of the most authoritative and recent methods. Dr. Bray's style of composition is at once direct and thorough, and his subject matter is extremely well organized, clear to the reader, and exhaustive.

In addition to regular methods the book includes special sections on the technique of basal metabolism tests, allergy tests, qualitative and quantitative methods for identification of poisons and foreign substances, surgical pathology, normal tables, preparation and use of indicators, and preparation of stains and reagents.

To the second edition have been added the many advances in clinical laboratory methods made during the past two years.

Among the additional procedures in this edition are serum phosphatase determination, titration of staphylococcus antitoxin in the blood serum, Ivy bleeding time, peroxidase-Giemsa staining method, cough plate method for diagnosis of whooping cough, the halo or diffraction method for measuring erythrocytes, determination of cyanates in the blood, 'phthalein elimination curve, determination of sulfanilamide in the blood, the differential heterophile agglutination for infectious mononucleosis, and determination of vitamin C.

---

\*Synopsis of Clinical Laboratory Methods. By W. E. Bray, B.A., M.D., Professor of Clinical Pathology, University of Virginia; Director of Clinical Laboratories, University of Virginia Hospital. Cloth, ed. 2, 324 pages, 51 illustrations, 17 color plates, \$4.50. The C. V. Mosby Co., St. Louis, Mo.

# *The Journal of Laboratory and Clinical Medicine*

VOL 24

MAY, 1939

No 8

## *CLINICAL AND EXPERIMENTAL*

### SULFANILAMIDE AND DERIVATIVES IN THE TREATMENT OF EXPERIMENTAL PNEUMOCOCCUS INFECTIONS\*

JOHN A. KOLMER, M.D., GEORGE W. RAUZZI, PH.D. ANNA M. RULE  
PHILADELPHIA, PA.

BECAUSE of the biologic relationship of the pneumococcus to streptococcus, sulfanilamide (para aminobenzenesulfonamide) and its derivatives have properly commanded considerable interest in relation to the treatment of experimental and human pneumococcus infections.

In 1936 Buttle and Gray<sup>1</sup> reported that sulfanilamide by oral administration had a slight effect upon type I pneumococcus infections of mice inoculated intraperitoneally, but only when very small infective amounts of the organism were employed. Later Buttle and his colleagues<sup>2</sup> found this likewise true of type II pneumococcus.

Rosenthal,<sup>3</sup> however, observed that sulfanilamide in olive oil administered to mice by subcutaneous injection afforded a considerable and encouraging degree of protection against types I, II, and III pneumococci inoculated intraperitoneally. Cooper, Gross, and Mellon<sup>4</sup> also found that the oral administration of the compound prolonged and sometimes saved the lives of mice inoculated subcutaneously with type III pneumococci, and in a subsequent series of investigations Gross and Cooper<sup>5</sup> reported that the compound by oral administration was effective in prolonging and sometimes saving the lives of rats with pneumococcal pneumonia produced by intratracheal injections of types I, II, and III pneumococci suspended in mucus. With type II the results were no more effective than treatment with serum alone, but with type I best results were observed among rats treated simultaneously with sulfanilamide and serum. Schmidt<sup>6</sup> has stated that subcutaneous injections

\*From the Research Institute of Cutaneous Medicine Philadelphia.  
Received for publication October 8 1938.

of the compound were effective in the treatment of mice inoculated intraperitoneally with type XIV.

We have also observed encouraging results in the treatment of experimental pneumococcus meningitis with sulfanilamide by oral, subcutaneous, and intrathecal administration.<sup>7</sup> Types I, II, and III produced acute meningitis in rabbits by intracisternal inoculation along with an associated septicemia usually fatal in from two to four days. Treatment of 60 animals, begun four hours after inoculation, did not result in the recovery of any, but apparently prolonged the lives of 24 for various periods. The intracisternal inoculation of monkeys with virulent types I, II, and III produced fatal meningitis with septicemia in twenty-four to thirty hours. Treatment of 10 animals with sulfanilamide, begun twenty-four hours after inoculation, resulted in the recovery of one (type III) and apparently prolonged the lives of 7 others.

#### PURPOSE OF INVESTIGATION

In the present investigation we have sought to determine the influence of sulfanilamide by oral, subcutaneous, intravenous, and a combination of oral and subcutaneous administration upon types I, II, and III pneumococcus infections of rats inoculated intraperitoneally. Our purpose was to select strains of moderate virulence with the hope that these, along with the higher natural resistance of the rat as compared with the mouse, would enable us to administer multiple doses as well as make heart blood cultures.

Our special purpose, however, was to determine the influence of sulfanilamide upon the intradermal pneumococcus lesions of rabbits so frequently accompanied by an associated septicemia produced by the method of Goodner.<sup>8</sup> We have found this experimental lesion very satisfactory not only for chemotherapeutic studies in pneumococcus infections<sup>9, 10</sup> but likewise for chemotherapeutic investigations in streptococcus infections of rabbits<sup>11, 12</sup> because the local lesions of hyperemia, edema, and necrosis not only can be seen and measured daily for clinical purposes, but likewise, and very importantly, can be readily cultured at daily intervals for determining possible local bactericidal effects. Since pneumococcus infections of human beings are predominantly and primarily of the fixed tissues, with secondary invasion and infection of the blood, we have long considered this experimental intradermal pneumococcus "pneumonia" of special value for determining the efficacy of chemical agents not only in the disinfection of the blood, but also, and even more importantly, in the disinfection of the fixed tissues. Kreidler<sup>13</sup> has already reported upon sulfanilamide by oral administration in the treatment of this lesion of rabbits produced by the intradermal injection of 0.1 c.c. of a 1:1,000 dilution of eighteen-hour broth culture of type I pneumococcus, stating that it favorably influenced the septicemia, reduced the fever, and resulted in the recovery of most of the animals.

We have likewise been able to include a study of the acetyl derivative of sulfanilamide as well as 4,4'-diamino-diphenylsulfone first reported upon by Buttle, Stephenson, Smith, and Foster<sup>14</sup> and its acetyl derivative introduced by Fourneau, Trefouel, Nitti, and Bovet.<sup>15</sup> Bauer and Rosenthal<sup>16</sup> have

found 4,4' diamino diphenylsulfone and its acetyl derivative more effective than sulfanilamide in pneumococcal infections of mice, but much less marked than against streptococci. Life was prolonged but few animals permanently survived the pneumococcal infection.

Recently Whitby<sup>17</sup> has reported 2-aminobenzenesulfonamide pyridine effective in the treatment of mice infected with types I, II, III, V, VII and VIII pneumococci and especially types I, VII and VIII. Evans and Gairdner<sup>18</sup> report that its use in the treatment of 100 cases of pneumonia resulted in a mortality of 8 per cent as compared with a mortality of 27 per cent in a control group of cases. We have not been able to include this compound in the present investigation but it is interesting to note in this connection that Kolmer, Brown, Raiziss, Rule, and Clemence<sup>19</sup> have observed particularly encouraging results with two pyridine compounds containing an amino group or an amino group with iodine and especially 2,2'-pyridyl sulfide dihydriobamide in the treatment of intradermal streptococcus infections of rabbits.

#### SULFANILAMIDE IN TREATMENT OF PNEUMOCOCCUS INFECTIONS OF RATS

In our first experiment 10 white rats weighing from 100 to 150 gm., were inoculated intraperitoneally with 0.2 cc. of an eighteen hour broth culture of type I pneumococcus, 10 additional animals with 0.2 cc. of a 1:5,000 dilution of type II, and 10 with 0.2 cc. of 1:600 dilution of type III.

Eight animals of each series were used for treatment with sulfanilamide, the first dose being given four hours after inoculation and thereafter two doses per day (10 A.M. and 3 P.M.) for four days in succession. Two animals of each series received 0.05 gm. per kg. orally, 2 the same dose subcutaneously,\* 2 intravenously, and 2 received 0.025 gm. per kg. orally with 0.025 gm. subcutaneously. The dose administered by all routes corresponded therefore, to 3.5 gm. per day per 70 kilograms.

TABLE I  
EFFECT OF SULFANILAMIDE ON TYPE I PNEUMOCOCCUS INFECTIONS OF RATS\*

DOSE PER KG. †				DAYS					
ORALLY	SUBCUT.	INTRAVEN.	ORALLY AND SUBCUTAN.	1	2	3	4	5	6
0.05				—†	—	D			
0.05				—	—	D			
	0.05			—	D	—	D		
	0.05			—	—	—	—	—	D
		0.05		—	—	—	D		
		0.05		—	—	—	—	—	
			0.025	—	—	D			
			0.025	—	—	—			
Control	—	—	—	—	D				
Control	—	—	—	—	D				

\*Inoculated intraperitoneally with 0.2 cc. of 1:60,000 dilution of eighteen hour broth culture.

†First dose four hours after inoculation thereafter twice daily (10 A.M. and 3 P.M.) for four days in succession.

— = survival D = died. All rats succumbing gave positive heart blood cultures.

As shown in Table I, the two controls inoculated with type I succumbed in forty eight to seventy two hours. Of the 8 treated animals the lives of only

\*0.8 per cent solution in water was employed for all parenteral administrations.

2, or possibly 3, were prolonged by one to three days, all animals ultimately succumbing with positive heart blood cultures.

The two controls inoculated with type II lived for three to five days. Of the 8 treated animals 3 survived the full period of fourteen days' observation and all of these had negative heart blood cultures at this time; the remaining 5 succumbed in two to four days with positive heart blood cultures.

TABLE II

EFFECT OF SULFANILAMIDE ON TYPE II PNEUMOCOCCUS INFECTIONS OF RATS\*

DOSE PER KG.†				DAYS													
ORALLY	SUB-CUT.	INTRA-VEN.	ORALLY AND SUBCUTAN.	1	2	3	4	5	6	7	8	9	10	12	14		
0.05				-†	D												
0.05				-	-	D	-	-	-	-	-	-	-	-	-		
	0.05			-	-	-	-	-	-	-	-	-	-	-	-		
		0.05		-	-	-	-	-	-	-	-	-	-	-	-		
		0.05		-	-	D											
			0.025	-	-	-	D										
			0.025	-	-	-	-										
			0.025	-	D	-	-										
			0.025	-	-	-	D										
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
Control	-	-	-	-	-	-	-	-	D								

\*Inoculated intraperitoneally with 0.2 c.c. of 1:5000 dilution of eighteen-hour broth culture.

†First dose 4 hours after inoculation; thereafter twice daily (10 A.M. and 3 P.M.) for four days in succession.

†- = survival; D = died. All rats succumbing gave positive heart blood cultures.

The two controls inoculated with type III succumbed in three days with positive heart blood cultures; of the 8 treated animals 4 survived four to eight days, but all ultimately succumbed with positive blood cultures.

TABLE III

EFFECT OF SULFANILAMIDE ON TYPE III PNEUMOCOCCUS INFECTIONS OF RATS\*

DOSAGE PER KG.†				DAYS							
ORALLY	SUBCUT.	INTRA-VEN.	ORALLY AND SUBCUTAN.	1	2	3	4	5	6	7	8
0.05				-†	D						
0.05				-	-	-	-	D			
	0.05			-	-	-	D				
		0.05		-	-	D	-	-	-	-	D
			0.025	-	-	-	-	-	-	-	
			0.025	-	-	-	-	D			
			0.025	-	-	-	-	-			
			0.025	-	-	D	-	-			
Control	-	-	-	-	-	D	-	-			
Control	-	-	-	-	-	D	-	-			

\*Inoculated intraperitoneally with 0.2 c.c. of 1:600 dilution of eighteen-hour broth culture.

†First dose four hours after inoculation; thereafter twice daily (10 A.M. and 3 P.M.) for four days in succession.

†- = survival; D = died. All rats succumbing gave positive heart blood cultures.

This experiment was then repeated with 20 rats inoculated intraperitoneally with 0.2 cc of 1:10,000 dilution of eighteen hour broth culture of type I, 20 inoculated with 0.2 cc of 1:600 dilution of type II and 20 with 0.2 cc of 1:600 dilution of type III.

Treatment was started four hours after inoculation followed by four additional treatments at daily intervals. Four rats of each series were kept as untreated controls, and of the remaining 16 animals 4 received 0.5 gm per kg orally, 4, 0.2 gm per kg subcutaneously 4 0.5 gm subcutaneously and the remaining 4, 0.5 gm orally with 0.2 gm subcutaneously per kg of weight. These amounts corresponded, therefore, to as much as 14 to 49 gm per 70 kg per day which were perfectly enormous amounts but still within the maximum tolerated doses for these animals.

As shown in Table IV, all of the four untreated controls inoculated with type I died in one to two days with positive blood cultures. Of the 16 treated animals the lives of 6 were apparently prolonged for one to two days, but all succumbed with positive heart blood cultures.

TABLE IV

EFFECT OF SULFANILAMIDE ON TYPE I PNEUMOCOCCUS INFECTIONS OF RATS\*

DOSAGE PER KG †			DAYS				
ORALLY	SUBCUT	ORALLY AND SUBCUTAN	1	2	3	4	5
0.5			-	D			
0.5			-	-	D		
0.5			-	D	-	D	
0.5			-	D			
	0.2		-	-	D		
	0.2		-	-	D		
	0.2		-	-	D		
	0.5		-	-	-	D	
	0.5		-	-	-		
	0.5		-	-	-	-	D
	0.5		-	-	-		
		0.5	-	D			
		0.5	-	D			
		0.2	-	-	D		
		0.5	-	-	-		
		0.2	-	-	-	-	D
Control	-	-	-	D			
Control	-	-	D	D			
Control	-	-	D	D			
Control	-	-	D	D			

\*Inoculated intraperitoneally with 0.2 cc of 1:10,000 dilution of eighteen hour broth culture.

†First dose four hours after inoculation thereafter daily for four days in succession.

- = survival D = died All rats succumbing gave positive heart blood cultures.

All of the 4 controls inoculated with type II died in two to six days with positive blood cultures. Of the 16 treated animals 11 survived for the full period of twenty days' observation at which time all gave negative blood cultures, the remaining 5 died in one to four days with positive blood cultures (Table V).

TABLE V  
EFFECT OF SULFANILAMIDE ON TYPE II PNEUMOCOCCUS INFECTIONS OF RATS\*

DOSAGE PER KG.†			DAYS																
ORALLY	SUBCUT.	ORALLY AND SUBCUTAN.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	18	20
0.5			-†	D	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5		-	-															

\*Inoculated intraperitoneally with 0.2 c.c. of 1:600 dilution of eighteen-hour broth culture.  
†First dose four hours after inoculation; thereafter daily for four days in succession.  
+ = survival; D = died. All rats succumbing gave positive heart blood cultures.





All of the 4 controls inoculated with type III succumbed in two to five days with positive blood cultures. Of the remaining 16 treated animals, 9 survived the full period of twenty days' observation at which time the heart blood cultures were sterile; the remaining 7 animals died in three to nine days with positive blood cultures (Table VI).

It would appear, therefore, that sulfanilamide in the amounts administered by these various routes had but very slight influence upon type I pneumococcus peritonitis and septicemia of rats, but that favorable and encouraging results were observed with type II and type III infections. Apparently subcutaneous injections with oral administration gave much better results than oral administration alone.

#### SULFANILAMIDE IN TREATMENT OF INTRADERMAL PNEUMOCOCCUS INFECTIONS OF RABBITS

In this experiment 10 rabbits were inoculated intradermally (shaven abdominal skin) with 0.2 c.c. of 1:30 dilution of eighteen-hour broth culture of type I pneumococcus, 10 with 0.2 c.c. of 1:30 dilution of type II, and 10 with 0.6 c.c. of 1:30 dilution of type III.

Twenty-four hours later well-developed local lesions of hyperemia and edema were present and, especially with the type II and III strains, at which time the first treatment was given. Subsequently the compound was administered twice daily (10 A.M. and 3 P.M.) for five days in succession, totaling ten treatments in the case of those animals surviving five days or longer.

Sulfanilamide was given orally to 2 rabbits in each series in dose of 0.05 gm. per kg. twice daily; 2 were given the same dose intramuscularly, 2 intravenously, and the remaining 2 of each series received 0.025 gm. orally with 0.025 gm. intramuscularly. These were equivalent to 0.1 gm. daily, corresponding to 7.0 gm. for an adult weighing 70 kg.

Cultures were made of each local lesion daily and heart blood cultures upon the death of the animals.

As shown in Table VII, the two untreated controls inoculated with type I died in two to four days, respectively, with positive lesion and blood cultures. All of the remaining 8 treated animals also succumbed with positive lesion and blood cultures, although the lives of 2 receiving the compound orally were prolonged for about three days.

The two controls inoculated with type II survived for the full period of seven days' observation at which time they showed positive lesion cultures. Of the remaining 8 treated animals 6 likewise survived, but the lesions of all showed definite clinical improvement and were sterile upon culture (Table VIII).

Both of the untreated controls inoculated with type III also survived the full period of seven days' observation at which time the local lesions were still severe with positive cultures. All of the 8 treated animals also survived and showed definite healing with negative lesion cultures in all except one (Table IX).

TABLE VII  
EFFECT OF SULFANILAMIDE UPON INTRADERMAL TYPE I PNEUMOCOCCUS LESIONS OF RABBITS\*

DOSE PER KO.†			FIRST DAY‡		SECOND DAY		THIRD DAY		FOURTH DAY		FIFTH DAY		SEVENTH DAY	
ORALLY	INTRAMUS.	INTRAVEN.	ORALLY AND INTRAMUS.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.
0.05				2**	+	3	+	4	+	4	+	4	+	D
0.05				2	+	4	+	4	+	4	+	4	+	D
	0.05			3	+	D	+							
	0.05			3	+	D	+							
		0.05		3	+	D	+							
		0.05		3	+	4	+	4	+	D	+			
			0.025											
			0.025	2	+	4	+	D	+					
			0.025	2	+	3	+	4	+	D	+			
			0.025	2	+	3	+	4	+	D	+			
Control	-	-	-	3	+	D	+							
Control	-	-	-	3	+	D	+							

\*Inoculated intradermally with 0.2 cc of 1:30 dilution of eighteen-hour broth culture

†Given twice daily (10 A.M. and 3 P.M.) for five days

‡Twenty-four hours after inoculation, treatment begun

\*\*Local lesion 1 = large area of hyperemia, 2 = hyperemia and edema, 3 = severe hemorrhagic lesion, 4 = very severe lesion with necrosis

D = died with positive blood cultures + = lesion culture positive - = lesion culture sterile

TABLE VIII  
EFFECT OF SULFANILAMIDE UPON INTRADERMAL TYPE II PNEUMOCOCCUS LESIONS OF RABBITS\*

ORALLY	DOSE PER KG.†			FIRST DAY‡		SECOND DAY		THIRD DAY		FOURTH DAY		FIFTH DAY		SEVENTH DAY	
	INTRAMUS.	INTRAVEN.	ORALLY AND INTRAMUS.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.
0.05				4**	+	4	+	4	+	4	+	2	+	2	-
0.05	0.05			4	+	4	+	4	+	4	+	3	+	D	+
	0.05			4	+	4	+	4	+	2	-	2	-	2	-
		0.05		4	+	D	+								
		0.05		4	+	4	+	4	+	2	+	2	+	2	+
		0.05		4	+	2	-	2	-	2	+	1	+	1	-
		0.025		4	+	3	-	3	+	2	+	2	-	1	-
		0.025		4	+	4	+	3	+	2	+	2	+	1	-
		0.025		4	+	4	+	4	+	2	+	3	+	3	+
Control	-	-		4	+	4	+	4	+	2	+	2	+	2	+
Control	-	-		4	+	4	+	4	+	2	+	2	+	2	+

\*Inoculated intradermally with 0.2 c.c. of 1:30 dilution of eighteen-hour broth culture.

†Given twice daily (10 A.M. and 3 P.M.) for five days.

‡Twenty-four hours after inoculation; treatment begun.

\*\*Local lesion: 1 = large area of hyperemia; 2 = hyperemia and edema; 3 = severe hemorrhagic lesion; 1 = very severe lesion with necrosis; D = died with positive blood cultures; + = lesion culture positive; - = lesion culture sterile.



TABLE X  
EFFECT OF SULFANILAMIDE AND DERIVATIVES UPON INTRADESMAL TYPE I PNEUMOCOCCUS LESIONS OF RABBITS\*

COMPOUNDS	DOSE PER KG.† (GM.)	FIRST DAY‡		SECOND DAY		THIRD DAY		FOURTH DAY		FIFTH DAY		SEVENTH DAY		TENTH DAY	
		CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.
Sulfanilamide	0.05	2**	+	4	+	4	+	D	+	D	+				
	0.125	2	+	4	+	4	+	4	+	D	+				
	0.250	2	+	4	+	4	+	4	+	D	+				
Acetyl derivative of sulfanilamide	0.05	2	+	4	+	D	+	4	+	4	+				
	0.100	2	+	3	+	3	+	4	+	D	+	D	+		
	0.150	2	+	2	+	4	+	4	+	D	+				
Diamino-diphenyl- sulfone	0.0125	2	+	2	+	D	+	3	+	2	+				
	0.025	2	+	3	+	4	+	4	+	2	+	2	+	1	-
	0.5	2	+	2	+	4	+	4	+	4	+	D	+		
Diacetyldiamino- diphenylsulfone	0.125	2	+	4	+	D	+	D	+						
	0.25	2	+	4	+	4	+								
	0.5	2	+	D	+										
Controls	-	2	+	4	+	4	+	D	+						
	-	2	+	4	+	3	+	D	+						
	-	2	+	3	+	4	+	D	+						

\*Inoculated intradermally with 0.2 c.c. of 1:30 dilution of eighteen-hour broth culture.  
†Orally twice daily (10 A.M. and 3 P.M.) for five days.  
‡Twenty-four hours after inoculation; treatment begun.

\*\*Locust lesion; 1 = large area of hyperemia; 2 = hyperemia and edema; 3 = severe hemorrhagic lesion; 4 = very severe lesion with necrosis;

D = died with positive heart blood cultures; + = lesion culture positive; - = lesion culture sterile.

The results have indicated, therefore, that sulfanilamide has a definite curative effect upon intradermal pneumococcus infections of rabbits and especially those produced by types II and III no essential differences were noted according to the route of administration

#### SULFANILAMIDE DERIVATIVES IN TREATMENT OF INTRADERMAL PNEUMOCOCCUS INFECTIONS OF RABBITS

In this experiment 15 rabbits were inoculated intradermally with 0.2 cc of eighteen hour broth culture of type I pneumococcus 15 with the same dose of type II, and 15 with the same dose of type III

All three strains produced large local lesions with positive cultures

Three animals of each series were kept as untreated controls, those inoculated with type I succumbing in four days, and those with type II in five to seven days, with positive lesion and heart blood cultures. The 3 inoculated with type III survived the period of ten days observation at which time abscesses had developed with positive cultures

The remaining 12 animals of each series were used for treatment, 3 being given sulfanilamide orally twice a day (10 A.M. and 3 P.M.) for five days in succession, totaling 10 doses in the case of those animals surviving six days or longer after inoculation, since the first dose was given twenty four hours after inoculation. Of these 3, one received 0.05 gm (0.1 gm daily), one 0.125 gm (0.250 gm daily), and one 0.250 gm (0.5 gm daily) per kilogram. Three animals of each series were treated orally with similar doses of the acetyl derivative of sulfanilamide, 3 with 4,4' diamino diphenylsulfone, and 3 with the acetyl derivative of 4,4' diamino diphenylsulfone

As shown in Table X, the 3 untreated controls inoculated with type I pneumococcus succumbed four days after inoculation with positive lesion and heart blood cultures as previously stated. The 3 treated with sulfanilamide died in four to five days, and the 3 treated with its acetyl derivative died in three to seven days. Of the 3 treated with 4,4' diamino diphenylsulfone however, one survived the full ten days with marked healing of the local lesion and a negative lesion culture while the remaining 2 died on the third and seventh days, respectively, with positive lesion and blood cultures. All 3 treated with the acetyl derivative of 4,4' diamino diphenylsulfone died in two to four days with positive lesion and blood cultures

The 3 untreated controls inoculated with type II pneumococcus died in five to seven days with positive lesion and blood cultures (Table XI). Of the 3 treated with sulfanilamide one lived the full period of 10 days at which time the local lesion had almost healed with a negative culture, the remaining 2 died five to ten days after inoculation with positive lesion and blood cultures. Of the 3 treated with its acetyl derivative 2 lived the full period of ten days at which time some healing of the local lesion had occurred but both showing positive lesion cultures

All 3 treated with 4,4' diamino diphenylsulfone survived for ten days at which time the lesions showed considerable healing, but still yielding positive cultures

TABLE XI  
EFFECT OF SULFANILAMIDE AND DERIVATIVES UPON INTRADERMAL TYPE II PNEUMOCOCCUS LESIONS OF RABBITS\*

COMPOUNDS	DOSE PER KG.† (G.M.)	FIRST DAY†		SECOND DAY		THIRD DAY		FOURTH DAY		FIFTH DAY		SEVENTH DAY		FIFTH DAY	
		CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.	CLIN.	CULT.
Sulfanilamide	0.05	4**	+	4	+	3	+	3	+	2	-	1	-	1	-
	0.125	4	+	4	+	3	+	2	+	D	+	2	+	D	+
	0.250	4	+	4	+	3	+	3	+	2	+	2	+	2	+
Acetyl derivative of sulfanilamide	0.05	4	+	4	+	4	+	4	+	4	+	2	+	2	+
	0.100	4	+	4	+	4	+	4	+	4	+	1	+	2	+
	0.150*	4	+	4	+	4	+	3	+	D	+	3	+	2	+
Diamino diphenylsul- fone	0.0125	4	+	4	+	4	+	4	+	2	+	2	+	2	+
	0.025	4	+	4	+	4	+	4	+	2	+	1	+	1	+
	0.5	4	+	4	+	4	+	4	+	3	+	1	+	1	+
Diacetyl-diamino di- phenylsulfone	0.125	4	+	4	+	4	+	4	+	2	+	2	+	1	+
	0.25	4	+	4	+	4	+	3	+	3	+	3	+	1	+
	0.5	4	+	4	+	4	+	4	+	3	+	2	+	2	+
Controls	-	4	+	4	+	4	+	4	+	D	+	D	+		
	-	4	+	4	+	4	+	4	+	4	+	4	+		
	-	4	+	4	+	4	+	4	+	D	+	D	+		

\*Inoculated intradermally with 0.2 c.c. of 1:30 dilution of eighteen-hour broth culture.

†Orally twice daily (10 A.M. and 3 P.M.) for five days.

‡Twenty-four hours after inoculation; treatment begun

\*Local lesion: 1 = large area of hyperemia; 2 = hyperemia and edema; 3 = severe hemorrhagic lesion; 4 = very severe lesion with necrosis.  
D = died with positive heart blood cultures; + = lesion culture positive; - = lesion culture sterile.

TABLE VII  
EFFECT OF SULFANILAMIDE AND DERIVATIVES UPON INTRADERMAL TYPE III PNEUMOCOCCUS LESIONS OF RABBITS\*

COMPOUNDS	DOSE mg kg	FIRST DAY†		SECOND DAY		THIRD DAY		FOURTH DAY		FIFTH DAY		SEVENTH DAY		EIGHTH DAY	
		CIN	CULT	CIN	CULT	CIN	CULT	CIN	CULT	CIN	CULT	CIN	CULT	CIN	CULT
Sulfanilamide	0.05	2**	+	+	+	+	+	2	+	1	+	1	-	-	-
	0.125	1	+	+	+	1	+	1	+	1	-	1	-	-	-
	0.250	3	+	+	+	2	+	1	-	1	-	1	-	-	-
Acetyl derivative of sulfanilamide	0.05	3	+	+	+	+	+	2	+	1	+	1	+	1	+
	0.100	2	+	+	+	2	+	2	+	1	-	Abs	-	Abs	-
	0.150	3	+	+	+	+	+	2	+	2	+	2	+	D	+
Diamino diphenyl sulfone	0.0125	1	+	+	+	2	+	2	+	1	-	1	-	1	-
	0.25	3	+	+	+	2	+	1	-	1	-	1	-	D	+
	0.5	3	+	+	+	2	+	1	-	1	-	1	-	D	+
Diacetyl diamino diphenylsulfone	0.0125	2	+	+	+	+	+	2	+	+	+	1	+	1	+
	0.25	2	+	+	+	2	+	2	+	-	-	1	+	1	+
	0.5	2	+	+	+	2	+	2	+	1	-	1	-	1	+
(controls)	-	2	+	+	+	+	+	3	+	Abs	+	Abs	+	Abs	+
	-	2	+	+	+	1	+	2	+	Abs	+	Abs	+	Abs	+
	-	3	+	+	+	2	+	Abs	+	Abs	+	Abs	+	Abs	+

\*Inoculated intradermally with 0.2 cc of 1:30 dilution of eighteen hour broth culture.

†Orally twice daily (10 A.M. and 3 P.M.) for five days.

‡Twenty four hours after inoculation treatment begun.

\*\*Local lesion 1 = large area of hyperemia 2 = hyperemia and edema 3 = severe hemorrhagic lesion 4 = very severe lesion with necrosis

D = die 1 with positive heart blood cultures + = lesion culture positive - = lesion culture sterile



The 3 treated with the acetyl derivative of 4,4'-diamino-diphenylsulfone also survived the full ten days and likewise with considerable healing of the local lesions, but all showing positive lesion cultures.

As previously stated, all 3 untreated controls inoculated with type III pneumococcus survived the period of ten days, but abscesses developed with positive cultures (Table XII).

All 3 treated with sulfanilamide likewise survived with complete healing of the local lesions and negative cultures. Of the 3 treated with its acetyl derivative one died on the tenth day with a positive lesion and blood culture, while the remaining 2 survived, one with a negative lesion culture.

Of the 3 treated with 4,4'-diamino-diphenylsulfone 2 survived ten days with complete or almost complete healing with negative lesion cultures, while the remaining animal died on the tenth day with a local lesion well healed but with a positive lesion and blood culture. All 3 treated with its acetyl derivative lived the full ten days with almost complete healing of the local lesions along with negative lesion cultures.

#### CONCLUSIONS

1. Sulfanilamide by oral, subcutaneous, intravenous, and combined oral and subcutaneous administration was only very slightly effective in the treatment of type I pneumococcus peritonitis and septicemia of rats. Much better results were observed in types II and III infections.

2. Sulfanilamide by oral, intramuscular, intravenous, and combined oral and intramuscular administration was only slightly effective in the treatment of type I intradermal pneumococcus infections and septicemia of rabbits. Much better results were observed with type II and type III infections.

3. The acetyl derivative of sulfanilamide was less effective than sulfanilamide itself in the treatment of types I, II, and III intradermal pneumococcus infections and septicemia of rabbits by the four routes of administration.

4. 4,4'-Diamino-diphenylsulfone was more effective than sulfanilamide in the treatment of type I and type II intradermal pneumococcus infections of rabbits by the four routes of administration, but probably slightly less effective in the treatment of type III infections.

5. The diacetyl derivative of 4,4'-diamino-diphenylsulfone was less effective than sulfanilamide and diamino-diphenylsulfone in the treatment of type I and type II intradermal pneumococcus infections of rabbits with septicemia by the four routes of administration, but about equal to these compounds in the treatment of type III infections.

#### REFERENCES

1. Buttle, G. A. H., and Gray, W. H.: Protection of Mice Against Streptococcal and Other Infections by p-Aminobenzenesulfonamide and Related Substances, *Lancet* 1: 1256, 1936.
2. Buttle, G. A. H., Parrish, H. J., McLeod, M., and Stephenson, D.: The Chemotherapy of Typhoid and Some Other Non-Streptococcal Infections in Mice, *Lancet* 1: 681, 1937.
3. Rosenthal, S. M.: Chemotherapy of Experimental Pneumococcus Infections, *Pub. Health Rep.* 52: 48, 662, and 685, 1937.
4. Cooper, F. B., Gross, P., and Mellon, R. R.: Action of p-Aminobenzenesulfonamide on Type III Pneumococcus Infections in Mice, *Proc. Soc. Exper. Biol. & Med.* 36: 148, 1937.

- 5 Gross P, and Cooper, F B Efficacy of p Aminobenzenesulfonamide in Experimental Type III Pneumococcus Pneumonias of Rats *Proc Soc Exper Biol & Med* 36 225, 535, 678, and 774, 1937
- 6 Schmidt, L H Use of Sulfanilamide in the Treatment of Type XIV Pneumococcus Infections in Mice, *Proc Soc Exper Biol & Med* 37 205 1937
- 7 Kolmer, J A, Rule, A M, and Weiner M L Sulfanilamide in the Treatment of Experimental Streptococcal and Pneumococcal Meningitis *Arch Otolaryng* 27 519, 1938
- 8 Goodner, K Experimental Intradermal Pneumococcus Infection in Rabbits *J Exper Med* 48 1, 1928, *Ibid* 54 817 1931
- 9 Kolmer, J A, and Rule, A M Goodner's Intradermal Pneumococcus Infection of Rabbits, *J Infect Dis* 57 46 1935
- 10 Kolmer, J A, and Rule, A M Chemotherapy of Intradermal Pneumococcus Infection of Rabbits Effects of Optochin and Other Quinine Compounds, *Proc Soc Exper Biol & Med* 32 89, 1934
- 11 Kolmer, J A, and Rule, A M Experimental Streptococcus Infections in Rabbits for Therapeutic Investigations, *J Lab & Clin Med* 22 1097 1937
- 12 Kolmer, J A, Brown, H B, Rule, A M and Warner M F Toxicity Therapeutic Activity, and Mode of Action of Sulfanilamide in Experimental Streptococcus Infections of Rabbits, *J Lab & Clin Med* 24 164 1938
- 13 Kreidler, W A Treatment of Pneumococcal Infections in Rabbits With Sulfanilamide, *Proc Soc Exper Biol & Med* 37 146 1937
- 14 Buttle, G A, Stephenson D, Smith S and Foster G E Treatment of Streptococcal Infections in Mice With 4:4 Diaminodiphenylsulfone *Lancet* 1 1331, 1937
- 15 Fourneau, E, Trefouel, J, Nitti, P, and Boret, D Action Antistreptococcique der dérivés sulfures organiques, *Compt rend Acad d sc* 204 1763 1937 *Ibid* 205 299 1937
- 16 Bauer, H, and Rosenthal, S M Some New Sulphur Compounds Active Against Bacterial Infections, *Pub Health Rep* 53 40, 1938
- 17 Whitby, L E H Chemotherapy of Pneumococcal and Other Infections With 2 p Aminobenzenesulfonamido Pyridine, *Lancet* 1 1210 1938
- 18 Evans, G M, and Gulsford, W F Treatment of Pneumonia With 2 p Aminobenzene sulfonamido Pyridine, *Lancet* 2 14 1938
- 19 Kolmer, J A, Brown, H, Raiziss G W, Rule A M and Clemence L W The Chemotherapy of Experimental Streptococcus Infections of Rabbits With Special Reference to Pyridine Compounds and Prontosil Soluble *J Pharmacol & Exper Therap* 61 253, 1937

## THE DETERMINATION OF SULFAPYRIDINE IN BLOOD\*

E G SCHMIDT, PH D, BALTIMORE MD

RECENT experimental evidence indicates that 2 (p aminobenzenesulfonamido) pyridine<sup>2,3</sup> or sulfapyridine<sup>4</sup> is a more promising chemotherapeutic agent in pneumonia therapy than sulfanilamide itself. Intelligent treatment with this drug will require a knowledge of its concentration in the blood.<sup>5,7</sup> In the present paper data are given on the determination of sulfapyridine in the blood by means of three methods which have been used successfully for sulfanilamide analysis: the Marshall<sup>8,11</sup> and the Proom<sup>12,13</sup> diazotization procedures and the naphthoquinonesulfonic acid method<sup>14,17</sup> of the author which can be performed on routine blood filtrates.

### METHODS OF ANALYSIS AND RECOVERY OF ADDED SULFAPYRIDINE

The general plan of approach was the same as that used by the author in previous work on sulfanilamide analysis.<sup>14,16</sup> To 18 c.c. portions of pooled oxalated, human blood were added 2 c.c. quantities of stock sulfapyridine†

\*From the Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore.

†Merck and Co. Inc. kindly furnished the sulfapyridine used in these experiments.

solutions of such strengths as to yield a series of bloods containing 1 to 20 mg. sulfapyridine per 100 c.c. The stock solution was prepared by dissolving 1 gm. of the drug in hot distilled water. This solution was cooled, then diluted to 1000 c.c. Each cubic centimeter contained 1 mg. sulfapyridine. After a few days some of the compound would crystallize; when this occurred the solution was discarded. The various standards for analysis were prepared from a stock solution which contained 0.1 mg. per c.c. and which remained clear and stable even in the cold.

In the Marshall method exactly 3 c.c. quantities of blood were laked with 21 c.c. of 0.05 per cent saponin solution and then deproteinized with 6 c.c. of a 20 per cent p-toluenesulfonic acid solution. To 10 c.c. of filtrate was added 1 c.c. of freshly prepared 0.1 per cent sodium nitrite, followed by 1 c.c. of the phosphate-sulfamate solution,<sup>11</sup> and then by 5 c.c. of alcoholic, heat-activated<sup>10</sup> dimethyl-alpha-naphthylamine. Appropriate standards were treated in a similar manner, and comparisons were made in the colorimeter at the end of ten minutes, although the color would remain stable for at least an hour. In the Proom modification 3 c.c. of blood were laked with 21 c.c. of water, followed by deproteinization with 6 c.c. of 15 per cent trichloroacetic acid. Ten cubic centimeters of this filtrate and an equal volume of the various standards were treated as in the Marshall procedure.<sup>11</sup> In the naphthoquinonesulfonic acid method 3 c.c. quantities of blood were laked with 24 c.c. of 0.12 N sulfuric acid, followed by 3 c.c. of 10 per cent sodium tungstate solution. A drop of 0.1 N hydrochloric acid was added to 10 c.c. portions of each filtrate and standards, followed by 1 c.c. of 0.05 per cent sodium-beta-naphthoquinone-4-sulfonate (prepared from a 0.5 per cent solution, which remains stable for at least a month in a cold, dark place). The tubes were placed in the dark for an hour, after which the orange colored solutions were matched in the colorimeter. Since the reagent itself is colored, correct values can be obtained only if the unknown and the standard used for comparison are nearly alike.

TABLE I

RECOVERY OF SULFAPYRIDINE ADDED TO BLOOD BY VARIOUS METHODS OF ANALYSIS

NUMBER OF DETERMINATIONS	SULFAPYRIDINE ADDED TO BLOOD MG./100 C.C.	RECOVERY BY											
		MARSHALL METHOD				PROOM-MARSHALL METHOD				NAPHTHOQUINONE-SULFONIC ACID METHOD			
		MG./100 C.C. BLOOD			PER CENT AVERAGE	MG./100 C.C. BLOOD			PER CENT AVERAGE	MG./100 C.C. BLOOD			PER CENT AVERAGE
		MAX.	MIN.	AVG.		MAX.	MIN.	AVG.		MAX.	MIN.	AVG.	
1	20.0			19.7	98.5			18.5	92.5			16.0	80.0
4	10.0	10.0	9.8	9.87	98.7	9.5	9.1	9.3	93.0	8.2	7.9	8.1	81.0
4	5.0	5.1	4.8	4.9	98.0	4.8	4.6	4.65	93.0	4.3	3.8	4.1	82.0
5	2.5	2.6	2.4	2.5	100.0	2.27	2.17	2.23	89.2	2.2	2.0	2.05	82.0
1	1.0			0.98	98.0			0.94	94.0			0.84	84.0
Average per cent		98.6				92.3				81.8			

The data in Table I indicate that excellent recovery (98 to 100 per cent) of added sulfapyridine was obtained with the Marshall method,<sup>11</sup> but that quantitative recovery was not secured on the trichloroacetic acid filtrates and that

8 per cent of the added sulfapyridine was adsorbed by the precipitated proteins. This fact was confirmed in the following manner. Ten cubic centimeters of sulfapyridine solution containing 1 mg. of the drug were diluted to 100 cc. with control trichloroacetic acid blood filtrate. Another 10 cc. portion was diluted to the same volume with water containing 18 cc. of 15 per cent trichloroacetic acid per 100 cc. Ten cubic centimeter quantities of each of these solutions gave exactly the same amount of color after the addition of the proper reagents.<sup>2</sup> This differs from the sulfanilamide experiments wherein quantitative recovery was always obtained from the trichloroacetic acid filtrates also. The data also show that 18 per cent of the added sulfapyridine was adsorbed by the protein tungstate precipitate, as compared with 10 per cent in case of sulfanilamide reported in a previous paper.<sup>1</sup> Since these losses are consistent however, regardless of the concentration of sulfapyridine in the blood correct values can be secured by dividing the obtained values by the factors 0.92 and 0.82, respectively.

TABLE II

COMPARATIVE ANALYSIS OF THE BLOOD OF PATIENTS UNDERGOING SULFAPYRIDINE THERAPY\*

MARSHALL METHOD ON p-TOLUENESULFONIC ACID FILTRATES	(A) PROOM MARSHALL METHOD ON TRICHLOROACETIC ACID FILTRATES	(A) 0.92	(B) NAPHTHOQUINONE SULFONIC ACID METHOD ON TUNGSTIC ACID FILTRATES	(B) 0.82
16.0			13.0	15.9
12.0		12.5	9.6	11.7
10.0	11.5	9.8	8.2	10.0
9.1	9.0	9.0	7.2	8.8
8.6	8.3	8.0	7.2	8.8
5.7	7.8	5.5	5.0	6.1
5.7	5.3	5.4	4.3	5.2
4.6	5.0	4.6	3.4	4.1
3.1	4.2	2.9	2.5	3.0
2.2	2.7	2.1	1.8	2.2
1.5	1.9	1.4	1.4	1.7
0.8	1.3	0.8	0.7	0.9
	0.7			

\*The values are given as mg. of sulfapyridine per 100 cc. of blood.

In Table II data are given on a series of bloods secured from patients undergoing sulfapyridine therapy. Again it is obvious that the values given by the Marshall procedure<sup>11</sup> on the trichloroacetic acid filtrates are slightly lower than those given by the same method on the p-toluenesulfonic acid filtrates. When these lower values are corrected for sulfapyridine loss, incidental to deproteinization, by the factor 0.92, values are secured which check well with the higher and more correct ones given by the regular Marshall method. The findings by the naphthoquinonesulfonic acid method are likewise low. When these findings are divided by the correction factor 0.82 in this case values are secured which also check closely with those given by the Marshall procedure.

## CONCLUSIONS AND SUMMARY

The sulfapyridine concentration of blood can be accurately determined by the recent Marshall method on p-toluenesulfonic acid filtrates. On trichloroacetic acid filtrates, however, this method gives values which are low by 8 per cent. The naphthoquinonesulfonic acid method on tungstic acid filtrates gives results

which are low by 18 per cent. However, correct values can be obtained in each case by the use of proper correction factors. The regular Marshall procedure has the advantage of greater color production, better proportionality, and probably greater accuracy. However, the naphthoquinonesulfonic acid method seems to fit in better with the routine hospital work. Thus, it is carried out on the regular Folin-Wu tungstic acid blood filtrates, requires fewer and cheaper reagents, and a shorter working time.

## REFERENCES

1. Whitby, L. E. H.: Chemotherapy of Pneumococcal and Other Infections With 2 (p Aminobenzenesulphonamido) pyridine, *Lancet* 1: 1210, 1938
2. Evans, G. M., and Gaisford, W. F.: Treatment of Pneumonia With 2 (p Aminobenzene sulphonamido) pyridine, *Lancet* 2: 14, 1938.
3. Paris Foreign Letter, Action of Sulfanilamide Pyridine on the Pneumococcus, *J. A. M. A.* 111: 2404, 1938.
4. Report of the Council, *J. A. M. A.* 112: 49, 1939.
5. Hobson, F. G., and MacQuaide, D. H. G.: Treatment of Meningococcal Meningitis With 2 Sulphanilyl-Amidopyridine (M. & B. 693), *Lancet* 2: 1213, 1938
6. Maegraith, B. G., and Vollum, R. L.: The Bacteriostatic Effects of Sulphonamide P, Soluseptasine and M. & B. 693, *Brit. M. J.* 2: 985, 1938.
7. Mayer, R. L.: Activité de l'alpha (p aminophényl sulfamido) pyridine dans les infections colibacillaire et staphylococcique de rat souris, *Compt. rend Soc de biol* 129 480, 1938
8. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: p Aminobenzenesulfonamide *J. A. M. A.* 108: 953, 1937.
9. Marshall, E. K., Jr.: Determination of Sulfanilamide in Blood and Urine, *Proc Soc Exper. Biol & Med.* 36: 422, 1937.
10. Marshall, E. K., Jr.: Determination of Sulfanilamide in Blood and Urine, *J. Biol Chem* 122: 263, 1937.
11. Marshall, E. K., Jr., and Litchfield, J. T., Jr.: The Determination of Sulfanilamide, *Science* 88: 85, 1938
12. Proom, H.: Estimation of Sulfanilamide in Blood and Other Body Fluids, *Lancet* 1 260, 1938.
13. Kamlet, J.: The Determination of Sulfanilamide in Blood, *J. LAB & CLIN MED* 23 1101, 1938
14. Schmidt, E. G.: The Determination of Sulfanilamide in Tungstic Acid Blood Filtrates by Means of Sodium  $\beta$  Naphthoquinone 4-Sulfonate, *J. Biol Chem* 122: 757, 1938
15. Schmidt, E. G.: The Determination of Sulfanilamide in Cerebrospinal Fluid by Means of Sodium Beta Naphthoquinone 4 Sulfonate, *J. LAB & CLIN. MED* 23: 648, 1938
16. Schmidt, E. G.: Further Observations on the Determination of Sulfanilamide in Blood *J. LAB & CLIN. MED* In Press.

# THE INFLUENCE OF THE WATER SOLUBLE GONADOTROPIC FACTOR OF PREGNANCY URINE ON THE TESTES OF THE NORMAL IMMATURE AND MATURE RAT\*

H. S. RUBINSTEIN, M.D., PH.D. AND A. ABRAHAM, A.B., M.D.  
BALTIMORE, MD

THE classic work of Zondek (1926) Smith and Engle (1927), Evans and others (1933) has shown that the anterior pituitary gland controls the gonads. Zondek (1931) has shown that in pregnancy enormous amounts of pituitary like hormone are excreted in the urine. This hormone he has called prolan. Since this earlier work, a huge literature has accumulated on the effects of prolan on the gonads. While most of these reports concern themselves with the effects upon hypophysectomized animals relatively few critical studies have been carried out on the normal subject. Furthermore, reports dealing with the effects of pregnancy urine extract (prolan) upon the male gonad are at present quite conflicting. For example some deny its effect upon the germinal epithelium (Engle, 1932, Molien D'Amour, and Gustavson, 1933, Robson and Taylor, 1933, Collip 1934) while others maintain that spermatogenesis is either accelerated or increased (Bionha and Simonnet, 1929, Borst, Dodelein, and Gastmorovic, 1930 Krause, 1930, Boeters, 1931). Moreover, Brosius and Schaffer (1933) and Lloyd (1936) have reported spermatogenic stimulation in the human adult.

In view of these conflicting reports, some of which stood in contradiction to the results of one of us (Rubinstein, 1938), in producing an increased spermatozoal output in normal human males with this substance, we decided to reinvestigate the problem.

For this purpose, 40 albino male rats of Wistar Institute stock from thirty to one hundred days old were studied. One half of these (20) served as controls. Both groups were further subdivided into those older than forty five days (age of normal testicular descent—Donaldson, 1924) and those younger. All animals were kept on a diet of Purina dog chow daily, with green vegetables added twice weekly. Water was constantly present. The test animals received daily intraperitoneal injections of the water soluble gonadotropic factor of pregnancy urine to a total of 25 rat units for the ten days. The animals were sacrificed twenty-four hours after the last injection. Body and testicular weights were recorded, and the gonads were studied grossly and microscopically.

## RESULTS

The extract in the doses used had no effect upon the body weights. Pre mature testicular descent, as previously reported (Rubinstein, 1934), was noted

\*From the Laboratory for Research Surgical Division Sinal Hospital Baltimore  
Received for publication October 4 1938

in all treated animals. In addition, it led to significant increase in testicular weights in both mature and immature groups as shown in Table I.

TABLE I  
BODY WEIGHTS AND TESTICULAR WEIGHTS OF ALL ANIMALS

BODY WEIGHT				TESTICULAR WEIGHT			
TEST ANIMALS	CONTROLS	DIFFERENCE	C. R.*	TEST ANIMALS	CONTROLS	DIFFERENCE	C. R.
Immature $48.0 \pm 1.93$	$52 \pm 2.54$	$-4 \pm 3.2$	1.2	$0.352 \pm 0.004$	$0.308 \pm 0.007$	$0.044 \pm 0.007$	6.3
Mature $130.0 \pm 5.82$	$122 \pm 4.59$	$8 \pm 5.8$	1.4	$1.996 \pm 0.084$	$1.701 \pm 0.043$	$0.295 \pm 0.094$	3.1

\*C. R. indicates the ratio of difference. Differences are considered as probably significant only if this ratio is 3 or more.

Histologically, the testes of the immature treated animals (Fig. 1A) showed a marked increase in interstitial tissue, a moderate hyperemia, a definite increase in the number of spermatogonia and spermatocytes, and an increased number of mitotic figures. Spermatids were occasionally present, but mature spermatozoa could not be seen. In contrast to this the immature control testes (Fig. 1B) showed interstitial tissue which was very scant and mitotic figures which were considerably less in number. Mature sperm were also absent here. In the epididymis of the immature test animals (Fig. 1C) could be seen clumps of immature sperm consisting of spermatocytes which were often in active mitosis. The epithelium was definitely secretory in character. No such cell groups could be seen in the normal immature control epididymis (Fig. 1D).

The testes of the adult test animals showed a marked hyperemia and an increase in interstitial tissue. In addition, there was a slight increase of mitotic figures among the spermatogonia and spermatocytes as compared to the controls (Fig. 2A and B). Since the mature group showed mature spermatozoa in both test and control rats, no striking difference could be observed in their epididymides.

#### DISCUSSION

Practically all reports agree that the water-soluble gonadotropic factor of pregnancy urine (P. U. extract) causes a marked increase in the interstitial tissue of mature and immature rats. Our findings in this respect are merely corroborative. In addition to this effect the germinal epithelium in our animals also showed evidence of germinal cell stimulation resulting in a marked proliferation of spermatogonia and spermatocytes. Although this effect was quite noticeable in younger animals, no mature sperm were seen in this group.

The mechanism of the action of pregnancy urine extract upon the germinal epithelium of the testis is probably secondary to the liberation of androgenic substance through its action upon the interstitial cells. One is led to this explanation for the following reasons: First, the administration of androgens alone will lead to precocious testicular descent without evidence of interstitial cell involvement. Hamilton (1937) has shown this to be true in the monkey, and the same result has been obtained in this laboratory in the rat. Secondly, growth and maturation of the accessory sex organs as well as maintenance results



Fig. 1.—Testes and epididymis of immature test and control rats. *A*, Testis of test animal showing increased interstitial tissue and mitotic figures. *B*, Control testis showing very little interstitial tissue and predominantly inactive germinal cells. *C*, Test epididymis showing clumps of testicular germ cells within the lumen. *D*, Empty lumina of the epididymis of the test animal.



when androgens are administered to the castrate or immature animal. Thirdly, testosterone propionate will bring about proliferative changes of the germinal epithelium in the normal immature animal as well as in the hypophysectomized rat (Nelson and Gallagher, 1936). Correlative evidence is adduced by the fact that this androgen as well as pregnancy urine extract results in an increased sperm count in normal human adults (Rubinstein and Kurland).

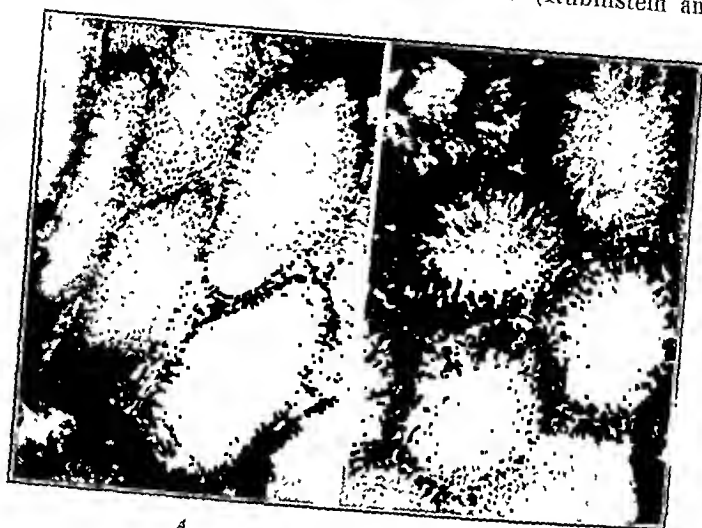


Fig. 2.—Testes of mature animals. A, Prolan treated animals showing increased interstitial tissue. B, Control.

Because the results enumerated above through the administration of male hormone (testosterone) can also be obtained with pregnancy urine extract which predominantly stimulates the interstitial tissue, and because it is now known that the interstitial tissue secretes the androgenic hormone, one may safely assume that the effects of pregnancy extract upon the germinal epithelium result secondarily to the liberated androgen.

#### CONCLUSIONS

The water-soluble gonadotropic factor of pregnancy urine injected into normal mature and immature rats leads to precocious testicular descent, significantly enlarged testes (by weight), and increased interstitial tissue. While this hormone stimulates germinal cell proliferation, it does not hasten maturation.

Its mode of action is discussed and it is assumed that while its effect upon the interstitial tissue is direct, its germinal proliferative activity is secondary to testicular androgenic influence.

#### REFERENCES

1. Boeters, H.: Das Hypophysenvorderlappenhormon (Prolan) und die männliche Keimdrüse, *Virchows Arch. f. path. Anat.* 280: 215, 1931.
2. Borst, M., Doderlein, A., and Gastimorovic, D.: Ueber die Einwirkung des Hypophysenvorderlappenhormons (Prolan) auf juvenile männliche Mäuse, *München. med. Wehnschr.* 77: 473, 1936, 1930.
3. Brosius, W. L., and Schaffer, R. L.: Spermatogenesis Following Therapy With the Gonad Stimulating Extract From Urine of Pregnancy, *J. A. M. A.* 101: 1227, 1933.

- 4 Brouha, L, and Simonnet, H Hormone antehypophysaire et tractus genitale male, *Ann de physiol* 5 562, 1929
- 5 Collip, J B Some Recent Advances in the Physiology of the Pituitary, *J Mt Sinai Hosp* 1 28, 1934
- 6 Donaldson, H H The Rat Memoirs of the Wistar Institute of Anatomy and Biology, No 6, Philadelphia, 1924
- 7 Eagle, E T The Action of Anterior Pituitary and of Pregnancy Urine on the Testes of Immature Rats and Monkeys, *Endocrinology* 16 506 1932
- 8 Evans, H M, Meyer, K, and Simpson, M E The Growth and Gonad Stimulating Hormones of the Anterior Hypophysis, Memoirs of the Univ of California, Vol 2, 1933
- 9 Hamilton, J B Production of Testicular Descent in the Monkey by Male Hormone Substance, *Am J Physiol* 119 325, 1937
- 10 Krause, E J Die Wirkung des Prolan (Aschheim Zondek) auf die mannliche geschlecht sorgino auf Grund von Versuchen an Maus und Ratte *Klin Wehnschr* 9 1493, 1930
- 11 Lloyd, V E Gonadotropic Hormone in the Treatment of Sterility in Man *Lancet* 1 474, 1936
- 12 Mohen, M, D'Amour, F E, and Gustafson R G Effects of Urinary Hebin Upon Immature Male Rats, *Endocrinology* 17 285, 1933
- 13 Nelson, W O Some Factors Involved in the Control of the Gametogenic and Endocrine Functions of the Testis Symposium on Quantitative Biology, Cold Spring Harbor 5 123, 1937
- 14 Nelson, W O, and Gallagher, T F Some Effects of Androgenic Substances in the Rat, *Science* 84 230, 1936
- 15 Robson, J M, and Tylor, H Factors Influencing the Functional Development of the Male, *Proc Roy Soc, London*, 8B 113 231, 1933
- 16 Rubinstein, H S The Effect of the Anterior Pituitary like Hormone on Spermatogenesis in the Human, *Endocrinology* 23 70, 1938
- 17 Rubinstein, H S The Production of Testicular Descent With the Water Soluble (Anterior Pituitary like) Fraction of Pregnancy Urine *Endocrinology* 18 475, 1934
- 18 Rubinstein H S, and Kurland, A The Effect of Testosterone Propionate on Spermatogenesis in the Human, *South M J* (In press)
- 19 Smith P E, and Engle, E T Experimental Evidence Regarding the Role of Anterior Pituitary in the Development and Regulation of the Genital System, *Am J Anat* 40 150, 1927
- 20 Zondek, B Ueber die Funktion des Ovariums, *Ztschr f Geburtsh u Gynak* 90 372, 1926
- 21 Zondek, B Die Hormone des Ovariums und des Hypophysenvorderlappens, Berlin 1931, Julius Springer

## EXCRETION OF VITAMIN C IN THE SWEAT\*

IRVING S. WRIGHT, A.B., M.D., AND ELIZABETH MACLEATHEN, B.S.,  
NEW YORK, N. Y.

EARLIER work, done by other workers and by ourselves, suggested that in persons who were febrile or who were subjected to temperatures sufficiently high as to cause an abnormal excretion of sweat, a substantial amount of vitamin C might be excreted through this excessive perspiration. Further studies along this line have failed to substantiate this belief.

Cornbleet, Klein, and Pace in 1936 reported that their determinations on sweat, collected from patients confined in a rubber sack during fever therapy, showed an average value of 0.55 to 0.64 mg. per cent ascorbic acid. Our figures have been considerably lower.

Sweat was collected from the face and shoulders of patients being treated in small whirlpool tanks and from the entire body of patients while under treatment in a hot circulation air cabinet at 105° F. The sweat was collected in small glass tubes and poured directly into iced tubes containing 1 c.c. of acetic acid. Five cubic centimeters aliquots were titrated with 2-6 dichlorophenolindophenol dye and the necessary corrections were made. In seventy-four determinations on 20 patients under the ordinary hospital dietary regime, the amount of ascorbic acid varied from 0.024 mg. per cent to 0.186 mg. per cent, the average being 0.041 mg. per cent. The lower figures were sometimes only very slightly higher than the water blank.

When the determinations showed the amount of ascorbic acid in the sweat to be exceedingly small, we began to question if the sweat was really an important avenue of loss of the vitamin from the body. To follow up this experiment, therefore, certain patients were given a five-hour test<sup>2</sup> a week prior to their fever therapy treatment, and the test was repeated again beginning five minutes prior to the start of the treatment. In this test the intravenous injection of 1,000 mg. of crystalline vitamin C was followed by a study of the urinary excretion of this substance during the succeeding five hours, according to the technique which we previously described. Sweat as well as the customary urines were collected. The five-hour urinary excretion during the treatment was lower than that of the week previous. However, as the titrations show, the diminished urinary return cannot be explained on the basis of loss through sweat. The perspiration collected over the five-hour period was divided roughly into four periods which corresponded to the rise and fall of the urinary excretion following an intravenous test

\*From the Department of Medicine of the Post-Graduate Medical School and Hospital, Columbia University.

Received for publication, October 10, 1938.

The crystalline vitamin C (cebone) used in this study was supplied through the kindness of Merck and Co., Inc.

dose of vitamin C. The results obtained from these separate specimens of sweat showed slight, if any, variation from each other, or from specimens collected from the patient before his test dose, or from the range of normal values. These 52 determinations varied from 0.029 to 0.057 mg per cent, and averaged 0.042 mg per cent as compared with the 0.041 mg per cent average value from persons on a normal diet. Since Gibson and Kopp have found that the gross water loss through the lungs and the skin during a five hour period under similar conditions ranges between 1,750 and 5,500 cc, the most vitamin C which would be lost (using our average figure) would be between 2 and 3 mg. This does not begin to compensate for the fall in the urinary excretion found when the five hour test is run simultaneously with a fever therapy treatment. It would, therefore appear that vitamin C is not lost in any appreciable amounts through excessive perspiration and that the lowered return in the urine is the result of increased body metabolism or some other factor.

#### CONCLUSIONS

1 The loss of vitamin C sustained during the spells of excessive sweating is probably best explained by an increased body metabolism and the subsequent increased utilization of ascorbic acid in the tissues.

2 The values obtained from samples of perspiration ranged from 0.024 to 0.186 mg per cent ascorbic acid. These values did not appear to be changed significantly by the introduction of large amounts of vitamin C (1 gm ceebione) intravenously.

#### REFERENCES

- 1 Cornbleet, Theodore, Klein, R. I., and Price, E. R. *Arch. Dermat. & Syph.* 34: 253, 1936.
- 2 Wright, I. S., Lilienfeld, A., and MacLennan, E. *Arch. Int. Med.* 60: 264, 1937.
- 3 Gibson, J. G., and Kopp, Israel. *J. Clin. Investigation* 7: 219, 1938.

## THE INTRADERMAL TEST FOR VITAMIN C DETERMINATION\*

IRVING S. WRIGHT, A.B., M.D., AND ELIZABETH MACLENATHEN, B.S.,  
NEW YORK, N. Y.

ROTTER in 1937, and later Portnoy and Wilkinson in the earlier months of 1938, described an intradermal test by which it was claimed vitamin C sub-nutrition could be determined quickly. They prepared a sterile solution of 2.6 dichlorophenolindophenol containing 2 mg. of the dye in 4.9 c.c. water. An area free of hair and superficial veins was cleansed with alcohol. One-hundredth cubic centimeter of dye was then injected intradermally, forming a wheal slightly larger than 2 mm. The time of injection and of complete disappearance of the dye coloration were carefully recorded. They found that in persons of normal vitamin C nutrition the average decoloration time was less than ten minutes, while in persons with a deficiency, the time for complete decoloration was above ten minutes.

In the series which we ran according to the above procedure, we were unable to obtain results which could be correlated with the dietary histories or the blood plasma ascorbic acid levels. Our results were studied from two angles: (1) the relationship of decoloration time to the dietary history and blood values for vitamin C, and (2) the effect of the site of injection on the decoloration time. Our observations are herewith presented.

1. *Decoloration Time on the Arm in Respect to Vitamin C Intake and Blood Level.*—In this series, the dietary history and the blood determinations (done according to the macromethod of Farmer and Abt) were closely related. The intradermal tests, however, showed slight, if any, correlation.

(A) Of 11 patients with an adequate intake and normal blood values, only 3 had a decoloration time of ten minutes or less. The others varied from eleven to nineteen minutes. According to the claims of Rotter, Portnoy, and others, all of these should average below ten minutes.

(B) In the group of patients with definitely poor dietary habits and with subnormal blood levels, 4 showed decoloration times of ten minutes or less. The remaining 7 took anywhere from eleven to thirty-three minutes.

2. *Decoloration Time and Site of Injection.*—While running the intradermal tests it was noted that there was frequently a wide variation in the response to tests injected within a few centimeters of each other on the volar surface of the same arm. The difference in the time on one occasion was as great as ten minutes. The question was then raised as to whether arterial incompetence or other peripheral vascular disabilities might not play a part

\*From the Department of Medicine of the Post-Graduate Medical School and Hospital, Columbia University.

Received for publication, October 10, 1938.

The crystalline vitamin C (ceebione) used in this study was supplied through the kindness of Merck and Co., Inc.

in any one person's response to an intradermal test of 2.6 dichlorophenolindophenol. Skin tests for vitamin C sufficiency or depletion were therefore, made on a group of patients in our vascular clinic. The injections were given on the volar surface of the lower arm and on the medial aspect of the calf of the leg. Where the arms were involved in a pathologic process, the tests were run on each arm and one leg. Where only the lower extremities were affected, the injections were given on each leg and one arm. Oscillometric readings were done on these patients. The following results were recorded:

(A) In one case there was a considerable discrepancy between the readings on each leg and on the right forearm.

(B) In 13 cases the readings on the different extremities agreed rather closely, i.e., within five minutes of each other.

(C) In 2 cases the decoloration times of one pair of extremities were approximately the same but differed from that on the opposite (lower or upper) extremity.

(D) In 4 cases the readings on one leg were in accord with those of the arm but differed from that of the other leg, the latter as a rule being longer. In this group it is interesting to note that the longer readings were frequently accompanied by diminished oscillometric reading below the knee.

All of the patients complained of an intense pain at the time of injection. This persisted for a few seconds. In several cases a hard erythematous nodule the size of the original wheal remained visible at the site of injection for four to five weeks.

#### CONCLUSIONS

We do not consider the intradermal injection of 2.6 dichlorophenolindophenol a reliable guide in the determination of the general status of the body vitamin C nutrition.

1. Variations in the decoloration time were too great to set up a normal range.

2. Readings taken at one site frequently varied considerably from those at either a close or distant site.

3. Cases of marked deficiency on two occasions fell within the normal set by Rotter, while the majority of our normals were above.

NOTE. An article by Poucher and Stubeurauch has appeared in the *J. Clin. Med.* since the completion of our series. These authors likewise report the inadequacy of the intradermal test.

#### REFERENCES

- Rotter, H. *Wien klin. Wchnsch.* 51: 205, 1908; *Nature*, London 139: 717, 1917.  
 Portnov, B., and Wilkinson, J. F. *Brit. M. J.* 1: 328, 1938.  
 Poucher, H. G., and Stubeurauch, C. F. *J. A. M. A.* 111: 303, 1938.

# POTASSIUM CYANIDE AS AN AGENT INHIBITING THE OXIDATION OF VITAMIN C IN VITRO\*

IRVING S. WRIGHT, A.B., M.D., AND ELIZABETH MACLENATHEN, B.S.,  
NEW YORK, N. Y.

THE report of Pijoan and Klemperer, that the addition of potassium cyanide to oxalated specimens of blood would prevent the oxidation of ascorbic acid in the blood plasma, was of great interest to us. Following their procedure (the addition of 5 mg. of potassium cyanide and 10 mg. of potassium oxalate for every 6 to 7 c.c. of blood), we ran a series of blood specimens in two batches, one with and one without the cyanide. After running over fifty such specimens, we have arrived at the conclusion that the addition of potassium cyanide does not increase the accuracy of the present method of determining the ascorbic acid values of blood plasma using the macromethod of Farmer and Abt.

All of our specimens were run immediately after the withdrawal of the blood. Where it was possible to obtain large samples, further titrations were done in six or twenty-four hours. In many cases determinations made at once were the same in both specimens. In others, the cyanide sample gave slightly higher results. When excess blood was titrated at later hours, the results were occasionally, but not uniformly, the same as in the original titration in the cyanide specimens. This was true, though not as frequent in the plain oxalated specimens, and pointed toward confirmation of the statement of Farmer and Abt that bloods differ in their loss of reduction power on standing. Where there was a drop over the six- or twenty-four-hour period, it was generally less in the cyanide specimen. This was, however, unpredictable and hence of no practical value.

## CONCLUSIONS

1. The addition of potassium cyanide retards slightly, but does not prevent, the oxidation of ascorbic acid in the blood specimens. Moreover, it tends to enhance the value of the plasma as shown by recent work.<sup>3</sup>

2. The apparent protection against oxidation processes is so unpredictable as to be of no practical value.

## REFERENCES

1. Pijoan, M., and Klemperer, F. J.: *J. Clin. Investigation* 16: 443, 1937.
2. Pijoan, M., and Eddy, E. J.: *J. Lab. & Clin. Med.* 22: 1227, 1937.
3. Farmer, C. J., and Abt, A. F.: *Proc. Soc. Exper. Biol. & Med.* 38: 399, 1938.
4. Friedman, G. J., Rubin, S. H., and Kias, W.: *Proc. Soc. Exper. Biol. & Med.* 38: 358, 1938.

\*From the Department of Medicine of the Post-Graduate Medical School and Hospital, Columbia University.

Received for publication, October 10, 1938.

# BIOCHEMICAL OBSERVATIONS IN HYPOGLYCEMIA INDUCED BY INSULIN

## II † BEHAVIOR OF BLOOD GASES IN REACTION TO THE HYPOGLYCEMIC STATE

EDWARD F. ROSLBERG M.D. ROCHESTER, MINN.

RECENTLY while treating patients with schizophrenia by means of insulin, I made a number of biochemical observations during the hypoglycemic periods. Observations of the effect of insulin on the level of the blood uric acid have already been reported.<sup>6</sup> Among my data are included a group of studies of the behavior of the gases of the blood during the hypoglycemic state. Some scattered reports on this subject are available in the literature, but they are contradictory and are not extensive. I am submitting these studies because there appears to be a need for more observations of these effects.

### METHOD

Venous blood was drawn from an elbow vein without aid of tourniquet and avoiding stasis. The specimen was collected in a sterile syringe containing about 5 cc. of sterile mineral oil which was free of air bubbles. The blood was immediately transferred to a small glass beaker containing a few crystals of potassium oxalate covered by a layer of sterile mineral oil. The blood was stirred immediately and rather gently with a small glass rod. These samples were analyzed† for oxygen and carbon dioxide content within thirty minutes after the withdrawal of the samples. For determination of the oxygen capacity the samples were equilibrated with room air and at room temperature by rotating in a separatory funnel until maximal exposure of the blood was certain. This required fifteen minutes or longer. The technique and apparatus used to determine the oxygen and carbon dioxide of blood samples were those described by Van Slyke and Neill. All determinations were made in duplicate simultaneously on two machines. Oxygen saturation of venous blood is determined by dividing the value for oxygen content by the oxygen capacity. These studies were conducted with schizophrenic patients who were otherwise "metabolically normal."

### OBSERVATIONS

If one administers a large dose of insulin and allows hypoglycemia to develop, the level of oxygen content of venous blood increases markedly. The situation with oxygen under these circumstances frequently reaches to a

\*From the Mayo Foundation, Rochester.

†The report of the first study in this series was not designated by a Roman numeral. Its title appears in the list of references appended to this report.

‡Abridgment of thesis submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of M.S. in Medicine.

Received for publication October 20, 1938.

§Analysis made by Miss Elizabeth MacLay in the laboratory of Dr. A. E. Osterberg.



TABLE I  
GASES IN VENOUS BLOOD IN RELATION TO HYPOLYCEMIA INDUCED BY INSULIN

DATE	CASE NUMBER	UNITS OF INSULIN AT 7:20 A.M.	TIME	CARBON DIOXIDE CONTENT	OXYGEN CONTENT	OXYGEN SATURATION	OXYGEN CAPACITY	BLOOD SUGAR*	COMMENT
3/17/37	1	60	7:25 A.M. 10:09 A.M.	Volumes per cent 52.9 55.6	Volumes per cent 13.1 16.2	Volumes per cent 67.9 84.6	Volumes per cent 19.3 Not done	Mg. per 100 c.c. 102.0 22.0	In hypoglycemic coma
3/30/37	2	140	7:20 A.M. 10:00 A.M.	57.6 52.2	11.7 21.2	61.5 92.6	19.1 22.9	117.6 47.1	Marked hypoglycemic symptoms
3/30/37	3	90	7:20 A.M. 10:00 A.M.	61.9 57.2	10.5 17.3	54.3 86.5	19.3 20.0	138.9 58.8	Marked hypoglycemic symptoms
4/12/37	2	160	7:29 A.M. 10:30 A.M. 11:30 A.M. 1:30 P.M.		10.7 19.8 150 gm. sucrose through nasal tube 5.54	54.2 87.7 23.95	19.88 22.58 23.14	94.3 31.4 100	In hypoglycemic coma Conscious--appears normal

\*Micromethod of Folin

point equaling that of arterial blood. Also during hypoglycemia, the capacity of venous blood to carry oxygen is regularly somewhat increased. The response of the carbon dioxide carrying capacity to hypoglycemia is not always the same. In some studies slight rises were detected, whereas in other instances the effect of hypoglycemia was to induce a slight fall (Table I).

In each of the four studies the appearance of hypoglycemia was accompanied by a substantial rise in the oxygen saturation and oxygen content of the venous blood. In Cases 2 and 3 considerable increases in the values for oxygen capacity also appeared. In study 4 (Case 2) observations made two hours after the termination of the hypoglycemic episode showed that the oxygen content and oxygen saturation had returned to normal values for venous blood. At this time the color of the venous blood had again become dark.

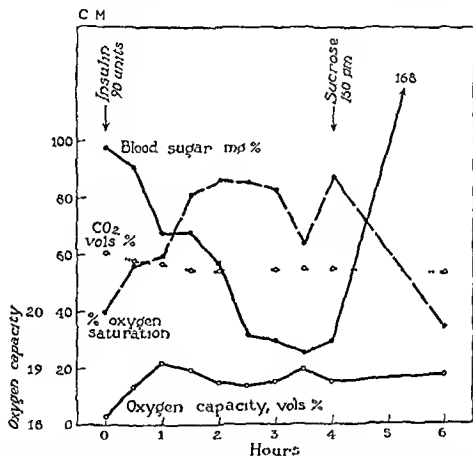


Fig 1—Gases in venous blood in relation to hypoglycemia induced by insulin. Development of the hypoglycemic state is accompanied by a marked rise in oxygen saturation, a rise in oxygen capacity, and a slight lowering of the level of carbon dioxide content.

The study on patient 3 illustrates the rates of change in the gas content of blood during hypoglycemia and establishes in the form of curves the speed of appearance and disappearance of these effects (Table II, Fig 1).

In this study ninety minutes after the injection of a large dose of insulin the oxygen content of venous blood had more than doubled, and this increase was maintained more or less constantly throughout the period of hypoglycemia. Carbohydrate was fed four hours after beginning the study. At the sixth hour when the patient was completely recovered from hypoglycemia the value for oxygen content and oxygen saturation had returned to normal. A definite rise in the oxygen carrying capacity of blood was likewise present one hour after the insulin injection. Not only was this increase maintained throughout the hypoglycemic period, it persisted and was present two hours

TABLE II  
GASES IN VENOUS BLOOD REPRESENTED IN FIG. 1

TIME AT WHICH BLOOD SAMPLE WAS TAKEN	CARBON DIOXIDE CONTENT	OXYGEN CONTENT	OXYGEN SATURATION	OXYGEN CAPACITY	BLOOD SUGAR*
	<i>Volumes per cent</i>	<i>Volumes per cent</i>	<i>Per cent</i>	<i>Volumes per cent</i>	<i>Mg. per 100 c.c.</i>
April 22, 1937. Patient 3; Insulin 90 Units at 7:15 A.M.					
7:15 A.M.	60.2	7.25	40.0	18.14	97.6
7:45 A.M.	58.0	9.40	56.3	18.64	90.9
8:15 A.M.	56.9	11.30	59.4	19.08	67.8
8:45 A.M.	54.9	15.20	81.2	18.96	67.8
9:15 A.M.	54.1	16.10	85.9	18.73	57.1
9:45 A.M.	54.4	16.00	85.7	18.67	51.2
10:15 A.M.	54.7	15.50	82.7	18.75	29.4
10:45 A.M.	54.9	12.30	64.8	18.98	25.0
11:15 A.M.	54.9	16.3	86.9	18.76	29.4
11:17 A.M.	150 gm. sucrose through nasal tube				
1:15 P.M.	53.2	8.45	44.8	18.87	168.0

\*Micromethod of Folin.

after administration of a large amount of carbohydrate had restored the blood sugar to normal and the patient to consciousness. Thus not all effects of insulin are counteracted promptly by carbohydrate feeding. The charted curve of the values for the carbon dioxide content of the blood shows a fall during the first two hours following the administration of insulin. This curve then reaches a level which is maintained throughout the remainder of the observation period. The regular form of this charted curve suggests that the change is not due to chance, but that it is the result of some definite change in blood chemistry as yet not understood.

*Control Observations.*—Two studies were made on patients of the same group under similar circumstances. The patients were in bed; one was allowed

TABLE III  
GASES IN VENOUS BLOOD: CONTROL OBSERVATIONS—NO INSULIN GIVEN

DATE	CASE NUMBER	TIME	CARBON DIOXIDE CONTENT	OXYGEN CONTENT	OXYGEN SATURATION	OXYGEN CAPACITY	BLOOD SUGAR*	COMMENT
			<i>Volumes per cent</i>	<i>Volumes per cent</i>	<i>Volumes per cent</i>	<i>Volumes per cent</i>	<i>Milli- grams per 100 c.c.</i>	
4/14/37	4	7:35 A.M.	56.8	7.1	36.1	19.4	117.6	Breakfast al- lowed at 7 A.M.
		10:00 A.M.	58.2	9.2	49.8	18.4	116.3	
		11:30 A.M.	150 gm. sucrose in 500 c.c. milk by mouth				125	
		1:30 P.M.	61.5	5.7	30.7	18.8	125	
4/15/37	3	7:00 A.M.	56.2	10.4	53.7	19.4	133.3	No breakfast allowed
		10:30 A.M.	55.0	13.7	67.0	20.5	124.3	
		11:30 A.M.	150 gm. sucrose in 500 c.c. milk by mouth				127.0	
		1:30 P.M.	55.3	14.0	70.9	19.7	127.0	

\*Micromethod of Folin.

breakfast and the other was deprived of food. One hundred and fifty grams of sucrose were administered to each by mouth at a time corresponding to the carbohydrate feedings in the above studies (Table III).

Contrasts between the findings in these control studies and those studies in which insulin was administered and hypoglycemia allowed to appear are obvious. No significant changes appeared in the oxygen capacity and the degree of saturation of the venous blood with oxygen remained within normal limits for venous blood in all observations on control subjects. The margin of error of the determination of oxygen capacity is usually taken to be 0.5 volume per cent. For this reason, the changes in oxygen capacity in the control study are thought to be insignificant, whereas the degree of change in oxygen capacity noted in the studies during hypoglycemia are thought to be significant.

#### COMMENT

In 1923 Olmsted and Logan studied the effect of insulin on the central nervous system and noted that arterial blood of cats was very dark and venous in color when the blood sugar was low. They stated that the approach of the blood sugar to the convulsive level could be foretold by observing the color and character of the blood. They also described "many points of resemblance between an insulin convulsion and an asphyxial convulsion such as that caused by clamping the trachea of a cat or rabbit." Later Olmsted and Taylor reported that the saturation of hemoglobin with oxygen remains constant for three hours after administration of a convulsive dose of insulin, that is, until one hour before a convulsion then it falls. In one of their experiments at the beginning of a convulsion the oxyhemoglobin fell to 75 per cent from an original level of 88 per cent and after the convulsions it returned to nearly normal. Klein and Holzer found that in hypoglycemia the alkali reserve, as measured by changes in the carbon dioxide combining power, did not vary beyond the limits of normal but patients in whom the more violent symptoms appeared such as sweating, palpitation, shivering, hunger thirst, and so forth, did show a slight lowering of the alkali reserve. They also noted that in contrast to the observations of Olmsted and Logan in rabbits, the red color of venous blood almost equals that of arterial blood during hypoglycemia in man. Later they<sup>3</sup> reported studies of the oxygen saturation of venous blood during hypoglycemia, using the Barcroft apparatus. They found that the oxygen saturation of venous blood in thirteen instances of hypoglycemia varied from 86 per cent to 97 per cent. Wiechmann and Koch also observed this color change as did Lauter and Bannann who noted also a slight increase in the oxygen capacity of the venous blood during hypoglycemia. Briggs, Koelug, Davis and Weber have stated that during hypoglycemia there occurs a rise in the value of lactic acid in the blood. This may account for the fall in carbon dioxide capacity which I observed in some of my studies.

The cause of the increased oxygen saturation of venous blood in hypoglycemia is not clear. One probable explanation is suggested by the observation of Ernstene and Altshule of an average of 29 per cent increase in cir-

culating blood volume per minute during hypoglycemia. If no alteration in oxygen utilization in the tissues occurs in hypoglycemic states, then with a greater circulating volume per minute less oxygen would be removed from a unit volume of blood and the venous blood could be expected to be more saturated with oxygen.

The increased carrying capacity of blood for oxygen during hypoglycemia is probably due to hemoconcentration. Further observations, including studies of behavior of the values for hemoglobin, hematocrit, erythrocyte count, plasma chlorides, and serum proteins, support this belief and will be reported later.

### CONCLUSIONS

1. The development of the hypoglycemic state is associated with a marked increase in the concentration of oxygen in the venous blood. This concentration reaches values usually found in studies of the arterial blood.

2. The blood has a greater oxygen-carrying capacity during hypoglycemia than normally.

3. In one instance a slight but steady fall occurred in the value for carbon dioxide content of venous blood.

### REFERENCES

1. Briggs, A. P., Koechig, Irene, Doisy, E. A., and Weber, C. J.: Some Changes in the Composition of Blood Due to the Injection of Insulin, *J. Biol. Chem.* 58: 721, 1924.
2. Ernestene, A. C., and Altschule, M. D.: The Effect of Insulin Hypoglycemia on the Circulation, *J. Clin. Investigation* 10: 521, 1931.
3. Holzer, H., and Klein, O.: Ueber die Sauerstoffsättigung des Venenblutes peripherer Gefäßgebiete im Insulinschock, *München. med. Wehnschr.* 2: 1284, 1928.
4. Klein, O., and Holzer, H.: Weitere Mitteilungen über Insulinhypoglykämie und Insulinschock beim Menschen, *Ztschr. f. klin. Med.* 107: 94, 1928.
5. Lauter, S., and Baumann, H.: Kreislauf und Atmung im hypoglykämischen Zustand, *Deutsches Arch. f. klin. Med.* 163: 161, 1929.
6. Olmsted, J. M. D., and Logan, H. D.: The Effect of Insulin on the Central Nervous System and Its Relation to the Pituitary Body, *Am. J. Physiol.* 66: 437, 1923.
7. Olmsted, J. M. D., and Taylor, A. C.: The Effect of Insulin on the Oxygen Saturation of Hemoglobin, *J. Biol. Chem. (Scientific Proceedings)* 59: 30, 1934.
8. Rosenberg, E. F.: Effect of Insulin on the Concentration of Uric Acid in the Blood, *J. Clin. Investigation* 17: 233, 1938.
9. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, *J. Biol. Chem.* 61: 523, 1924.
10. Wiechmann, Ernst, and Koch, Fritz: Ueber die Sauerstoffsättigung des Venenblutes peripherer Gefäßgebiete im Insulinschock, *München. med. Wehnschr.* 2: 1800, 1928.
11. Wiechmann, Ernst, and Koch, Fritz: Untersuchungen über den hypoglykämischen Zustand nach Insulininjektion. III. Mitteilung. Das Verhalten des Kreislaufs im hypoglykämischen Zustand, *Deutsches Arch. f. klin. Med.* 163: 176, 1929.

# BIOCHEMICAL OBSERVATIONS IN HYPOGLYCEMIA INDUCED BY INSULIN\*

## III GLYCORRHACHIA AND HYPOGLYCEMIA†

EDWARD F. ROSENBERG M.D. ROCHESTER MINN.

MANY outstanding clinical phenomena of the hypoglycemic state are manifestly due to disturbances within the central nervous system. These symptoms presumably reflect alterations in the chemistry of the nerve tissues. Studies of the behavior of constituents of the cerebrospinal fluid might be expected to throw some light on the nature of the changes which occur in the brain, yet so far as I am aware, only one paper dealing with experimental effects of insulin hypoglycemia on the cerebrospinal fluid of man and only a few reports of the results of animal experiments have been published. Accordingly, I am submitting the following data which bear on this subject.

### METHOD

Lumbar puncture and venipuncture were performed on fasting patients at about 7 A.M. and samples were removed for analyses. Following this a "coma dose" of insulin was injected. Thereafter until hypoglycemia was terminated by administration of sucrose, lumbar puncture and venipuncture were repeated hourly. Determinations were also made during the afternoons following termination of coma, until the sugar values of the cerebrospinal fluid had returned to approximately their original level. All patients used in these studies were schizophrenics in whom, except for functional mental disturbance, there was no evidence of disease.

### OBSERVATIONS

If one administers a large amount of insulin and induces a profound fall in the level of the blood sugar, the cerebrospinal fluid sugar level also falls, though not so rapidly as does the blood sugar (Table I, Fig. 1).

For the purposes of a control I made the following observations. A patient was kept at rest in bed. No food and no insulin were given in the morning (Table II). Thus in a fasting patient to whom no insulin is administered, the sugar content of the cerebrospinal fluid varies somewhat as does the blood sugar during the course of the day. These variations are small and have no definite pattern.

The level of chlorides in the cerebrospinal fluid in the second study (Table I) was not influenced by the effects of the insulin. In the third study (Table I) the afternoon chloride levels were somewhat lower than those made

\*From the Mayo Foundation, Rochester.

†Abridgment of thesis submitted to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of M.S. in Medicine.

Received for publication October 20, 1933.

TABLE I  
STUDIES OF CEREBROSPINAL FLUID IN RELATION TO HYPOGLYCEMIA

DATE	INSULIN UNITS GIVEN AT 7 A.M.	CASE NUMBER	TIME	BLOOD SUGAR*	CEREBROSPINAL FLUID					WATER PRESSURE		COMMENT
					SUGAR*	CELLS	NONNE	PROTEIN	CHLO- RIDES†	BEFORE	AFTER	
1/28/37	70	1		<i>Mg. per 100 c.c.</i>	<i>Mg. per 100 c.c.</i>			<i>Mg. per 100 c.c.†</i>		<i>Cm.</i>	<i>Cm.</i>	
			7 A.M.	112	79.5							In coma
			8 A.M.	77.6	75.5							In coma
			9 A.M.	25.6	67.5							In coma
			10 A.M.	21.6	65.6							
			11 A.M.	25.1	42.5							
2/27/37	70	1	11:05 A.M.	150 gm. sucrose in 500 c.c. water through nasal tube								Awake
					46.3							Awake
			1 P.M.	148	65.6							Awake
			3 P.M.	127.5	74.5							
			5 P.M.	130.0								
			7 A.M.	97.1	80	4 Lymphs.‡ Few R.B.C.	+	45	726	15	8	
			8 A.M.	86.0	75.2	5 Lymphs. Few R.B.C.	+	50	726	8	6	
			9 A.M.	31.2	65.8	Bloody	+	100	720	7	6	In coma
			10 A.M.	29.0	38.1	5 Lymphs. Few R.B.C.	+	50	722	8	6	In coma
			11 A.M.	20.7	31.4	4 Lymphs. Few R.B.C.	+	60	722	8	6	In coma
			11:05 A.M.	150 gm. sucrose in 500 c.c. water through nasal tube								
					32.2	10 Lymphs. Few R.B.C.	+	60	722	8	6	Awake
			1 P.M.	137	80.9	15 Lymphs. Few R.B.C.	+	60	726	8	6	Awake
			3 P.M.	119.7								
			5 P.M.	89	51			Not done		10	9	Awake

TABLE I—CONT'D

2/ 5/37	70	2	7 A M 8 A M 9 A M 10 A M 11 A M 11 05 A M	108.1 90.1 26.0 17.7 19.2 150 gm sucrose in 500 cc water through nasal tube	70 11.3 33 45 32	720 726 720 726 723	In coma In coma In coma
			1 P M 3 P M 5 P M	186.9 156.2 133.3	47.6 51.3 83.3	660 687 Not done	Awake Awake Awake
5/ 5/37	100	3	7 45 A M 8 45 A M 9 45 A M  10 45 A M 12 30 P M 12 35 P M  1 45 P M 3 45 P M	95 73 57  23 7 150 gm sucrose in 500 cc water through nasal tube 129 141	68 58 34  25 23  40.5 54.0		Severe hypo glycemic symptoms In coma In coma  Awake

\*Micromethod of Iolin

†Kingsbury Clark Williams and Post method\*

‡Osterberg Schmidt modification of Whiteborn method\*

§Lymphs = lymphocytes RBC = erythrocytes



during the hypoglycemic period. These differences probably resulted from the fact that patient 2 sweated profusely during hypoglycemia and doubtless lost chlorides in perspiration, whereas patient 1 remained dry throughout the studies

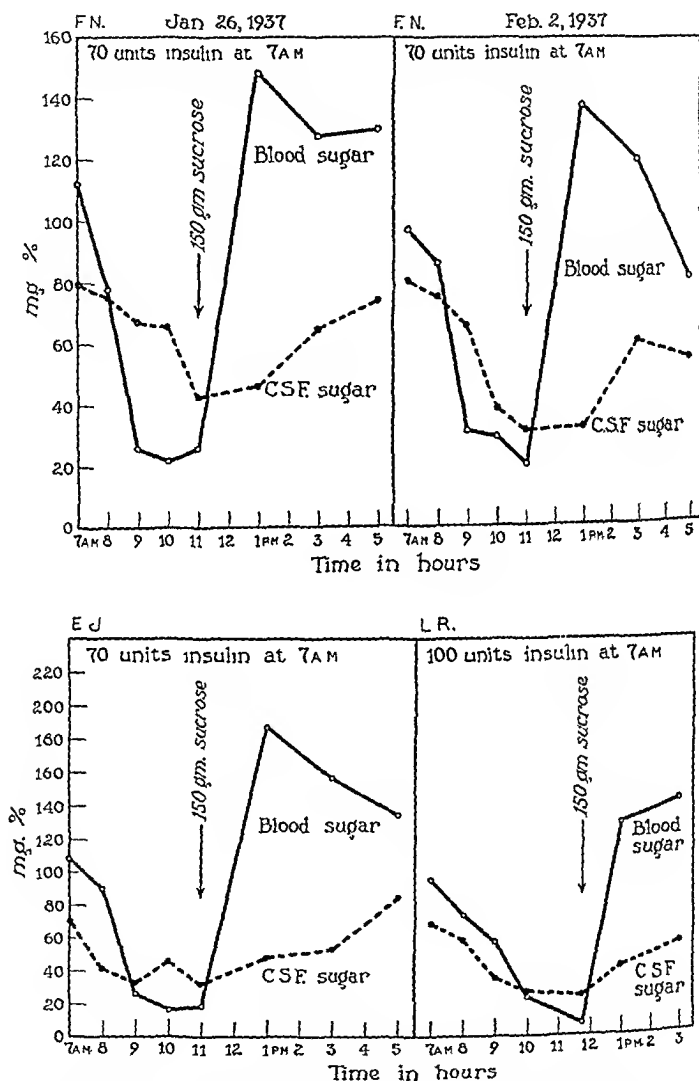


Fig. 1.—Studies illustrating the relationship between the level of *d*-glucose in the blood and in cerebrospinal fluid during hypoglycemia induced by insulin.

In the second study (Table I) there does not appear to be any significant alteration in the level of protein in the cerebrospinal fluid as a result of insulin administration and no pressure changes attributable to the action of insulin. The erythrocytes probably resulted from trauma.

#### COMMENT

Rathery and Sigwald observed the effect of insulin hypoglycemia on the sugar content of the cerebrospinal fluid of dogs. They stated that in hypo-

glycemia the level of sugar in the cerebrospinal fluid dropped but not so far relatively as did the blood sugar. In one instance blood sugar fell from 120 mg per 100 cc to 37 mg per 100 cc, and the cerebrospinal fluid sugar fell from 80 mg per 100 cc to 54 mg per 100 cc. In the second instance blood sugar fell from 86 mg per 100 cc to 26 mg per 100 cc, the cerebrospinal fluid sugar falling from 87 mg per 100 cc to 43 mg per 100 cc. As to the pressure of the cerebrospinal fluid in hypoglycemia they always found a relative hypotension during the hypoglycemic period as compared with the original pressure.

TABLE II

CONTROL STUDY OF CEREBROSPINAL FLUID IN RELATION TO BLOOD SUGAR

TIME SAMPLES WERE TAKEN	BLOOD SUGAR*	CEREBROSPINAL FLUID SUGAR*
	Mg per 100 cc	Mg per 100 cc
7 A M	95	57
9 30 A M	88	62
11 30 A M	95	57
12 Noon	"lunch"	
1 45 P M	11	65
3 45 P M	122	68

\*Micromethod of Folin

Cahane stated that there is a close relationship between the quantity of sugar in blood and in the cerebrospinal fluid. Lowering of the level of the blood sugar appeared half an hour after administration of insulin. One hour after the injection the level of sugar in the cerebrospinal fluid was lowered. The fall in level of the cerebrospinal fluid sugar was relatively less than that of the blood sugar. These observations were made on 4 human beings and 2 dogs, each subject was studied after administration of 20 units of insulin. No observations of the recovery phase were reported.

Kasahara and Uetani have reported studies of the cerebrospinal fluid sugar in the rabbit following insulin administration. Insulin doses of 0.5 unit per kilogram induced hypoglycemia in the rabbit which attained its maximum between the second and third hours after the injection. The level of the cerebrospinal fluid sugar usually had returned to normal after seven hours.

Numerous individual observations of the level of the cerebrospinal fluid sugar of patients in hypoglycemia have undoubtedly been studied and some have been reported. Davis and Brown have published such a study. They observed a patient in hypoglycemic coma and administered 100 gm of *d*-glucose intravenously, but the patient did not respond. Blood sugar after death was 176 mg per 100 cc but the spinal fluid sugar was only 25 mg per 100 cc. Then they made some studies in dogs. First they recorded the blood and cerebrospinal fluid sugar values and then administered insulin. When the dogs were in hypoglycemia, these values were redetermined. Insulin caused the usual fall in blood sugar and the cerebrospinal fluid sugar fell also. The drop in blood sugar was more marked than the drop in sugar level of the cerebrospinal fluid.

Diabkin and Ravdin found a rise in the pressure of the cerebrospinal fluid of hydrated dogs during hypoglycemia, but no rise in the pressure of the

cerebrospinal fluid in dehydrated dogs under the same circumstances. Patient 1 in my second study (Table I) did not have any perceptible rise during hypoglycemia in the pressure of the cerebrospinal fluid as measured by the water manometer.

#### CONCLUSIONS

1. Lowering of the level of blood sugar following the administration of insulin is accompanied in man by a corresponding but slower fall in level of cerebrospinal fluid sugar.

2. Administration of carbohydrate to a patient in hypoglycemic coma induces a rapid rise in blood sugar level and a corresponding but slower rise in level of cerebrospinal fluid sugar.

3. Administration of insulin has no definite influence on the level of chlorides or protein in cerebrospinal fluid.

4. No changes in pressure of cerebrospinal fluid which can be attributed to the effect of insulin occurred during insulin hypoglycemia among these patients.

#### REFERENCES

1. Cahane, M.: Influence de l'insuline sur la glycorachie, *Compt. rend. Soc. de biol.* 111: 111, 1932.
2. Davis, Reuben, and Brown, Herman: Spinal Fluid Sugar Determinations in Experimental Hypoglycemia of Dogs, *J. LAB. & CLIN. MED.* 19: 1049, 1934.
3. Drabkin, D. L., and Ravdin, I. S.: The Mechanism of Convulsions in Insulin Hypoglycemia. Interrelationship of Blood Concentration, Cerebrospinal Pressure and Convulsions, *Am. J. Physiol.* 118: 174, 1937.
4. Kasahara, Michio, and Uetani, Nikichi: The Effect of Insulin Upon the Reducing Substance in the Cerebrospinal Fluid of Normal Rabbits, *J. Biol. Chem.* 59: 433, 1924.
5. Moersch, F. P., and Kernohan, J. W.: Hypoglycemia; Neurologic and Neuropathologic Studies, *Arch. Neurol. & Psychiat.* 39: 242, 1938.
6. Osterberg, A. E., and Schmidt, Edna V.: The Estimation of Plasma Chlorides, *J. LAB. & CLIN. MED.* 13: 172, 1927.
7. Rathery, F., and Sigwald, J.: Etude expérimentale des modifications humérales dans l'hypoglycémie insulinaire chez le chien, *Compt. rend. Soc. de biol.* 107: 1074, 1931.
8. Todd, J. C., and Sanford, A. H.: *Clinical Diagnosis by Laboratory Methods*, ed. 8, Philadelphia, 1935, W. B. Saunders Co., p. 544.

# PLATELET STUDIES IN NORMAL MEN AND WOMEN (MENSTRUATING AND NONMENSTRUATING) AND SUBJECTS WITH BLEEDING DISORDERS\*

## COUNTS, DISINTEGRATION RATES, AND INTRADERMAL PLATELET INJECTIONS

PEARL LEE, M.D., AND BETTA NIMS ERICKSON, M.A. DETROIT, MICH.

THE role played by the platelets in the mechanism of blood clotting has long furnished investigators with an intriguing problem. Extensive research over many years has yielded important facts concerning their origin, morphology, and physiology. More recently accurate knowledge of their chemical composition has been added.<sup>1</sup> Nevertheless, the exact manner in which they enter into the normal clotting process and the cause of their variations in the bleeding disorders is still very imperfectly understood.

The lack of adequate methods has in most instances, limited the clinical study of platelets to an enumeration of the total count. Recently, Olef<sup>2</sup> has evolved a method for making total counts which provides for a differential on the basis of size. Probably the best method available for determining the relative fragility of the platelets in health and disease<sup>3,7</sup> has also been developed by Olef.<sup>8†</sup> He styles his determination the "Disintegration Rate." The greatest advantage of this method is that it makes possible a comparison of the differential formula of the initial count with that obtained after the disintegration period.

Using these improved methods of approach to platelet behavior, a study was made of total and differential platelet counts and disintegration rates accompanied by platelet lipid determinations.<sup>9</sup> Normal men and women, menstruating women, hemophiliacs, and patients with thrombopenic purpuras were subjects of study. The results of this study are supplemented in this paper by the results of several years' work on skin sensitivity to washed platelet suspensions. The skin sensitivity is correlated with the total platelet count and disintegration rate.

Olef's method<sup>2</sup> for total and differential platelet counting is time consuming and requires considerable experience in its use for the production of successful results. The method is an indirect one, involving the use of a solution of sodium metaphosphate to prevent clumping and disintegration. A drop of the metaphosphate is placed over the site of the finger puncture so that the blood is mixed directly with the diluting fluid and does not contact the skin.

\*From the Children's Hospital of Michigan and the Research Laboratory, Children's Fund of Michigan.

Received for publication October 21, 1938.

†Olef's technique for disintegration rate<sup>8</sup> was modified slightly by placing the paraffin cup containing the blood in the incubator at 37° C for six hours instead of the refrigerator for eight hours.

TABLE I

## TOTAL AND DIFFERENTIAL PLATELET COUNTS\*

(All platelet counts expressed in thousands)

SUBJECTS			NUMBER OF COUNTS	TOTAL PLATELET RANGE	COUNT† NEAREST MEAN	COUNT NEAREST MEAN DIFFERENTIAL COUNT					
NO.	AGE	SEX				GROUP I		GROUP II		GROUP III	
						per cent		per cent		per cent	
14 Normal Men and Nonmenstruating Women											
1	31	M	3	788-870	800	56	7	648	81	96	12
1	31	M	3	369-458	427	192	45	222	52	13	31
2	22	M	5	850-1030	957	57	6	766	80	134	14
2	22	M	6	520-793	626	319	51	301	48	6	1
3	32	M	6	480-690	570	188	33	342	60	40	7
3	32	M	2	490-570	490	245	50	240	49	5	1
4	27	M	4	477-570	508	137	27	371	73	0	
5	23	F	2	440-693	440	114	26	326	74	0	
6	31	F	5	328-712	424	84	20	280	66	60	14
6	31	F	4	542-813	715	350	49	365	51	0	
6	31	F	6	302-421	371	152	41	219	59	0	**
7	27	F	3	720-928	811	219	27	560	69	32	4
8	30	F	2	511-586	511	128	25	383	75	0	
9	42	F	3	629-882	828	248	30	580	70	0	
9	42	F	4	350-407	378	159	42	215	57	4	14
9	42	F	2	423-448	423	288	68	135	32	0	
10	25	F	2	574-613	574	86	15	465	81	23	4
11	25	F	4	578-801	693	249	36	367	53	76	11
12	24	F	4	585-873	792	340	43	428	54	24	3
13	31	F	3	1026-1206	1098	318	29	758	69	22	2
14	27	F	3	639-761	711	242	34	455	64	14	2
Total counts			76								

## 5 Normal Women, Menstruating

5	23	F	4	400-539	428	111	26	287	67	30	7
5	23	F	2	539-621	539	140	26	377	70	22	4
6	31	F	3	481-502	485	218	45	267	55	0	
7	27	F	4	387-582	461	157	34	300	46	4	1
8	30	F	3	426-471	451	221	49	230	51	0	
9	42	F	2	504-558	504	222	44	257	51	25	5
9	42	F	2	330-688	330	122	37	208	63	0	
Total counts			20								

\*All counts in each group were taken within thirty days. Thirty days or more elapsed between groups of counts on the same individual.

†When only two counts were taken, the lowest total was used as count nearest mean.

‡Acute cold and sinusitis for ten days.

§Sinus infection and severe cough. Five days elapsed between this and the preceding period.

\*\*Acute cold and sinusitis.

After transferring the mixture to a paraffin cup, fresh preparations are made for counting. A simultaneous erythrocyte count is also necessary, to calculate the absolute number of platelets. The platelets are differentiated into three classes according to size, using the red blood cell as a standard. Group I is one-fourth the size of the red blood cell, Group II is one-third, and Group III is one-half. This method gives higher total counts than most of those used, mainly because there is less platelet destruction involved in the preparation of the slides. Further details of this method may be found in the author's paper.<sup>2</sup>

All blood samples were taken between 10 and 11 o'clock in the morning, approximately two hours after eating. To establish a normal total figure for

any individual, counts were made on three successive days. A variation of 100,000 was allowed as the limits of error.

Observations were made on 4 normal men, 10 normal women, when not menstruating, and 9 hemophilic children. Five of the women were studied during menstruation. The normal individuals were all in good health and had no history of unusual bleeding. The hemophiliacs had been under observation for several years. Their histories and laboratory and clinical findings were typical of this bleeding disorder.

Table I presents the normal total and differential studies. It consists of the counts divided into 21 groups, made on 14 different subjects. The count nearest the mean for the group is given with its differential. This method of selection is followed in all the tables. When only two counts were taken, the lowest is used with its differential.

Inspection of the total counts (Table I) reveals that the groups for the same individual vary widely. This bears out Blakefield's report<sup>11</sup> of wide variations from week to week upon the same subject. The counts in any group, with one exception, were taken within intervals of thirty days. Where there was a marked drop, it was usually associated with an acute cold and sinusitis. Subjects 1, 6, and 9 demonstrate these findings. There are no differences apparent between the men and women. Seven groups of counts presented in Table I were made upon 5 of the normal women when they were menstruating.

TABLE II  
TOTAL PLATELET AND DIFFERENTIAL COUNTS  
HEMOPHILIACS  
(All platelet counts expressed in thousands)

MALE SUBJECTS		NUMBER OF COUNTS	TOTAL PLATELET RANGE	COUNT NEAREST MEAN*	COUNT NEAREST MEAN DIFFERENTIAL COUNT					
NO.	AGE				GROUP I		GROUP II		GROUP III	
						per cent		per cent		per cent
15	13	3	450-1188	748	524	70	210	28	14	2
16	7	2	546-640	546	202	37	344	63	0	-
17	8	2	252-506	252	126	50	101	40	25	10
18	4	4	991-1157	1013	597	59	416	41	0	-
19	9	2	944-974	944	604	65	331	35	9	1
20	13	1	450	450	270	60	186	37	14	3
21	15	1	414	414	166	40	248	60	0	-
22	6	2	717-1441	317	101	32	216	68	0	-
23	8	1	639	639	256	40	377	59	6	1
Total counts		16								

\*When only two counts were taken the lowest was used.

The same type of information is given for hemophiliacs in Table II. Here a total of 16 counts is divided into nine groups. Nine different individuals are the subjects of the study.

A comparative analysis of Tables I and II may be made by comparing the group averages and ranges for the counts nearest the mean according to the frequency of the occurrence of the total counts at three levels (400-600 thousand, 600-800 thousand, and over 800 thousand). These figures are given in Table III.

It may be seen that about 63 per cent of the normal total counts are included at the lowest level. All the seven groups made on menstruating women are in the 400-600 thousand range. In this small series, the counts during menstruation are definitely lower than in the group including both men and nonmenstruating women. Genell<sup>12</sup> has pointed out similar decreases in the counts during menstruation.

TABLE III

AVERAGES AND RANGES OF DIFFERENTIAL PLATELET COUNTS OF TOTAL COUNTS AT THREE RANGE LEVELS

	NUMBER OF SUBJECTS	GROUPS OF DETERMINATIONS	FREQUENCY DISTRIBUTION ACCORDING TO TOTAL COUNT LEVELS		
			400-600 THOUSAND	600-800 THOUSAND	800+ THOUSAND
<i>Normal M &amp; F (Nonmenstruating)</i>	14 (4M, 10F)	21	11	5	5
Group I:					
Average			161	300	180
Range			84-288	242-350	56-318
Group II:					
Average			291	383	662
Range			135-465	301-455	560-766
<i>Normal F* (Nonmenstruating)</i>	5	9	6	1	2
Group I:					
Average			154	350	234
Range			84-288		219-245
Group II:					
Average			260	365	570
Range			135-383		560-580
<i>Normal F* (Menstruating)</i>	5	7	7	0	0
Group I:					
Average			170		
Range			111-222		
Group II:					
Average			275		
Range			208-377		
<i>Hemophiliacs† (Children)</i>	9	9	5	2	2
Group I:					
Average			173	390	600
Range			101-270	256-524	597-604
Group II:					
Average			215	294	374
Range			101-344	210-377	331-416

\*Same 5 women.

†Two counts in this group are below 350,000.

No significant difference between the different types of subjects is found in the distribution of Groups I and II, in the lowest total count level. However, there is a definite difference in the highest level counts (800,000 or over) for the normals and the hemophiliacs. In the high counts for the normals a much greater percentage of Group II is present than in the 400-600 thousand class. The hemophiliacs reverse this picture, i.e., they show a marked increase in the percentage of Group I in the high counts.

Olef<sup>13</sup> states in his paper on the clinical significance of the differential platelet count that in the study of 45 adults he obtained the following average Group I, 18.6 per cent, Group II, 63.3 per cent, Group III, 17.4 per cent. The method as used here gave averages of 33 per cent for Group I, and 63 per cent for Group II. Group III was usually so small that it has been ignored in the general averages.

Olef included a Group IV, consisting of odd shaped platelets, in the differential. The number of these is so small that we have ignored his group completely.

#### DISINTEGRATION RATES

In Table IV the disintegration rates for normal women are presented. The subjects when not menstruating show a decrease in total count after six hours, ranging from 30 per cent to 52 per cent with an average of 41. The women when menstruating have disintegration rates varying from -16 to +39, with an average of +2. (The "+" sign indicates that the total count was increased by that per cent.)

Table V presents 15 disintegration rates performed on 9 different hemophiliacs. The rates ranged from -27 to +89 with an average of +2.5. An analysis of the changes in the differential at the end of the six hour period is given in Table VI, based upon the results contained in Tables IV and V.

A comparison of the disintegration rate for the various subjects shows that the rate is much faster for the normal nonmenstruating women than for any of the other groups presented. The range was from -30 per cent to -52 per cent, with an average of -41 per cent. In this paper we have considered anything slower than -30 to be retarded. Olef<sup>8</sup> regarded any figure faster than -10 as normal. However, the method as handled in this laboratory gave much higher results. Perhaps incubation for six hours instead of placing in the refrigerator for eight hours, accounts for the difference. The analysis of the percentage changes in Groups I and II of the differential after the six hour period can be used to show general trends only, because of the small size of the groups.

The hemophiliacs are separated according to clotting time and treatment. While the untreated hemophiliacs tend to give a decrease in Group I (average -25 per cent) and an increase in Group II (average +63 per cent), the transfused cases present the reverse—that is, an increase in Group I and a decrease in Group II. This is, perhaps, a step toward the normal distribution. The one hemophiliac treated with placental coagulant and the one treated with splenic extract and also the three with shorter clotting times, show the same type of variation as the transfused group.<sup>14</sup>

Several facts are apparent from this analysis. First, the menstruating women and all the hemophiliacs have a markedly delayed platelet disintegration rate, second, the differential changes are important as well as the actual disintegration rates. All the hemophiliacs vary considerably from the normal, but the untreated cases with long clotting times show the widest variance. The decrease in Group I with an increase in Group II suggests that the smaller



TABLE IV  
DISINTEGRATION RATES—NORMAL WOMEN  
(All platelet counts expressed in thousands)

CASE NO.	CLOTTING TIME <sup>10</sup>		TOTAL PLATELETS	DIFFERENTIAL COUNT		
				GROUP I	GROUP II	GROUP III
<i>Nonmenstruating women</i>						
5	10 min.	Initial	693	173	499	21
		After 6 hr. incubation	431	147	263	21
		Increase or decrease	-38%	-15%	-47%	
6	12 min.	Initial	302	184	118	0
		After 6 hr. incubation	210	57	153	0
		Increase or decrease	-30%	-69%	+30%	
8	9 min.	Initial	586	88	445	53
		After 6 hr. incubation	282	102	163	17
		Increase or decrease	-52%	+16%	-63%	
9	11 min.	Initial	828	248	580	0
		After 6 hr. incubation	494	213	281	0
		Increase or decrease	-40%	-14%	-52%	
		Initial	643	315	328	0
		After 6 hr. incubation	347	111	205	31
		Increase or decrease	-46%	-65%	-38%	
<i>Menstruating women</i>						
5	19 min.	Initial	426	111	298	17
		After 6 hr. incubation	592	142	414	36
		Increase or decrease	+39%	+28%	+39%	
		Initial	621	168	453	0
		After 6 hr. incubation	522	214	303	5
		Increase or decrease	-16%	+27%	-33%	
6	17 min.	Initial	485	218	267	0
		After 6 hr. incubation	413	124	252	37
		Increase or decrease	-15%	-43%	-6%	
7	19 min.	Initial	461	157	300	4
		After 6 hr. incubation	417	258	158	0
		Increase or decrease	-10%	+64%	-47%	
8	15 min.	Initial	451	221	230	0
		After 6 hr. incubation	466	140	307	19
		Increase or decrease	+3%	-37%	+33%	
9	20 min.	Initial	504	222	237	25
		After 6 hr. incubation	513	308	205	0
		Increase or decrease	+2%	+39%	-20%	
		Initial	330	122	208	0
		After 6 hr. incubation	374	104	225	45
		Increase or decrease	+13%	-15%	+8%	

platelets have imbibed fluid and thus increased in size. Ferguson<sup>15</sup> has shown that the first stage in disintegration of the normal platelet is an absorption of fluid and consequent increase in size. Perhaps the process has stopped at this point instead of going on to complete breakdown or, in those subjects showing an increase in Group I, the process may have gone a step farther and resulted in the breaking up of the large forms into two or more smaller parts.

It seems obvious that transfusion and the treatment by splenic extract and placental coagulant have modified the abnormal differential somewhat, but not enough to change the disintegration rate. Clinical records indicate that

TABLE V  
DISINTEGRATION RATES—HEMORRHAGIC  
(All platelet counts expressed in thousands)

CASE NO.	CLOTTING TIME <sup>10</sup>		TOTAL PLATELETS	DIFFERENTIAL COUNT		
				GROUP I	GROUP II	GROUP III
<i>No treatment</i>						
15	2 hr.	Initial	748	524	210	14
		After 6 hr. incubation	545	262	261	22
		Increase or decrease	-27%	-50%	+24%	
		Initial	150	129	117	4.5
		After 6 hr. incubation	520	140	380	0
		Increase or decrease	+16%	-57%	+225%	
		Initial	1188	297	891	0
		After 6 hr. incubation	918	40	569	9
		Increase or decrease	-21%	+14%	-36%	
16	2½ hr.	Initial	610	65	269	6
		After 6 hr. incubation	498	201	289	3
		Increase or decrease	-22%	-44%	+7%	
17	1½ hr.	Initial	506	253	248	5
		After 6 hr. incubation	524	194	330	0
		Increase or decrease	+4%	-23%	+33%	
		Initial	252	126	101	25
		After 6 hr. incubation	177	167	305	5
		Increase or decrease	+89%	+12%	+202%	
18	2 hr.	Initial	1157	47	810	0
		After 6 hr. incubation	1124	180	944	0
		Increase or decrease	-3%	-48%	-16%	
21	17 min.	Initial	411	166	248	0
		After 6 hr. incubation	423	106	288	29
		Increase or decrease	+2%	16%	+16%	
22	18 min.	Initial	317	101	216	0
		After 6 hr. incubation	315	101	230	1
		Increase or decrease	1.5%	0%	+6%	
23	30 min.	Initial	639	256	377	6
		After 6 hr. incubation	530	159	366	5
		Increase or decrease	-17%	-18%	-3%	
<i>Transfusion</i>						
18		Initial	991	515	476	0
		After 6 hr. incubation	923	551	369	0
		Increase or decrease	-7%	+8%	-22%	
18		Initial	1114	345	758	11
		After 6 hr. incubation	1048	597	451	0
		Increase or decrease	-6%	+73%	-40%	
22		Initial	1441	476	922	43
		After 6 hr. incubation	1584	776	760	48
		Increase or decrease	+10%	+63%	-18%	
<i>Placental coagulant</i>						
19	4 hr.	Initial	974	127	847	0
		After 6 hr. incubation	991	268	723	1
		Increase or decrease	+2%	+111%	-15%	
<i>Splenic extract</i>						
20	5 hr.	Initial	450	270	167	13
		After 6 hr. incubation	515	412	98	5
		Increase or decrease	+14%	+52%	-41%	

TABLE VI

COMPARISON OF DIFFERENTIAL PLATELET COUNTS AFTER 6-HOUR DISINTEGRATION PERIOD

	AVG. DISINTEGRATION RATE	NUMBER OF SUBJECTS	GROUPS OF DETERMINATIONS	INCREASE AFTER 6 HOURS		DECREASE AFTER 6 HOURS		AVG. PERCENTAGE INCREASE OR DECREASE
				NUMBER	AVERAGE	NUMBER	AVERAGE	
				<i>Per cent</i>		<i>Per cent</i>		
<i>Normal F (Non-menstruating)</i>	-41	4	5					
Group I				1	16	4	41	-20
Group II				1	30	4	50	-34
<i>Normal F (Menstruating)</i>	+2	5	7					
Group I				4	40	3	32	+9
Group II				3	27	4	26	-3
<i>Hemophiliacs (no treatment)</i>	4.8	4	7					
Group I				2	23	5	44	-25
Group II				5	98	2	26	+62
<i>Hemophiliacs (Transfused)</i>	-1	2	3					
Group I				3	48	-	-	+45
Group II				-	-	3	27	-27
<i>Hemophiliac (treated with placental extract)</i>	+2	1	1					
Group I				1	111	-	-	+111
Group II				-	-	1	15	-15
<i>Hemophiliac (treated with splenic extract*)</i>	+14	1	1					
Group I				1	52	-	-	+52
Group II				-	-	1	41	-41
<i>Hemophiliacs with maximum clotting time of 30"</i>	-3	3	3					
Group I				(One showed no change)		2	37	-25
Group II				2	11	1	3	+6

\*A protein-free splenic extract prepared by Wilson Laboratories, Chicago.

the treatment in the two cases made the bleeding less severe. The cases with short clotting times have the same type of differentials as the treated cases. This similarity in platelets may indicate the cause of their shorter clotting time.

It is very interesting that the menstruating individuals should also show a retarded disintegration rate. In the small series here no definite conclusions can be drawn concerning the differential distribution. Their clotting times fall within the same range as the mild hemophiliacs.

## SENSITIVITY TO INTRADERMAL PLATELET INJECTIONS

For the past four years skin sensitivity tests with platelet preparations have been performed on subjects with normal clotting mechanisms, on hemophiliacs, and on patients with thrombopenic purpuras.

To prepare the platelet suspension, 10 c.c. of venous blood was drawn into a syringe containing 5 c.c. of 10 per cent sodium citrate. This was discharged into a flask containing another 5 c.c. of the citrate solution making the final concentration 5 per cent. During the last year Olef's metaphosphate solution has been substituted for the citrate since it seems to be a better platelet preservative. The blood was thoroughly chilled before centrifuging, and kept as cold as possible.

For injection the final washing of the suspension is performed with 0.85 per cent sodium chloride, and the platelets finally suspended in 3 c.c. of the saline. Sterile technique must be observed throughout.\*

Table VII represents the results of intradermal injection of 0.1 c.c. of the sterile platelet suspension. The normal reaction consists of a wheal about  $\frac{1}{8}$  inch in diameter which can be definitely felt on palpation with an area of redness  $\frac{1}{4}$  to  $\frac{3}{8}$  inch in diameter. Anything larger is considered to be abnormal and is designated as a hypersensitivity. Patients giving no reaction are designated as hyposensitive.

Nine subjects with normal clotting mechanisms and normal platelet counts are presented in Table VII. These give the reaction considered to be normal.

Eight untreated hemophiliacs gave absolutely negative reactions. This finding is very interesting when considered in the light of the retarded platelet disintegration rate of these patients as given in Table V.

Subjects 31 through 36 are patients with thrombopenic purpuras, proved by history and prolonged observation to be of the chronic idiopathic type. In these cases an increased reaction was observed.

Subjects 37 through 41 are patients with secondary thrombopenias following measles. These may be divided into two classes. One class gave an increased, the other a negative, reaction during the purpura. Both returned to normal as the platelet count returned to normal.

An aplastic anemia case (Subject 42) gave a negative skin test. This patient may be assumed to have had an aplasia of the platelets.

The question of the interpretation of skin sensitivity to platelets and its use in clinical diagnosis becomes an interesting one. Three types of reactions have been demonstrated: the one occurring in the normal individual, an increased reaction occurring in the idiopathic thrombopenias and part of the secondary types, and the absolutely negative test demonstrated thus far only in hemophilia and a few secondary thrombopenias.

What determines sensitivity or the lack of it? It is known that all the blood components, including the platelets, are constantly being destroyed and replaced. Probably in the normal individual enough substances are released into the circulating blood, from the disintegrating platelets, to produce a slight skin sensitivity. The extreme opposite of the normal is represented by the idiopathic thrombopenia. Here the platelets are probably being destroyed rapidly, thus releasing an excessive amount of platelet substances into the blood. The fact that most of these cases after splenectomy have shown

\*The lipid distribution of human platelets in health and disease.\*

TABLE VII  
SENSITIVITY TO INTRADERMAL PLATELET INJECTIONS

SUBJECT			DIAGNOSIS	PLATELET COUNT*	SKIN SENSITIVITY TO PLATELETS	DISINFECTION RATE
NO.	AGE	SEX				
5	23	F	Normal	693	Normal	-38
9	42	F	Normal	828	Normal	-40
24†	8	F	Mastoiditis	300	Normal	
25	4	M	Epilepsy	300	Normal	
26	5	F	Empyema	300	Normal	
27	6	F	Mastoiditis	250	Normal	
28	9	M	Mastoiditis	300	Normal	
29	12	M	Tuberculosis	250	Normal	
30	30	F	Normal	300	Normal	
15	13	M	Hemophilia	1188	Hypo	22
16	7	M	Hemophilia	640	Hypo	22
17	8	M	Hemophilia	506	Hypo	3+
18	4	M	Hemophilia	1157	Hypo	7+
19	9	M	Hemophilia	974	Hypo	01
20	13	M	Hemophilia	450	Hypo	14+
21	15	M	Hemophilia	414	Hypo	2+
23	6	M	Hemophilia	639	Hypo	17
Thrombopenic purpura:						
31	10	M	Chronic idiopathic	50	Hyper	
32	11	M	Chronic idiopathic	20	Hyper	
33	8	M	Chronic idiopathic	20	Hyper	
34	10	F	Chronic idiopathic	20	Hyper	
35†	11	F	Chronic idiopathic	24	Hyper	
36	5	M	Chronic idiopathic	49	Hyper	
37	2	F	Secondary thrombopenia	20	Hyper	Post merkle
37	2	F	Secondary thrombopenia (20 days later)	230	Normal	
38†	8	F	Secondary thrombopenia	19	Hyper	Post merkle
38†	8	F	Secondary thrombopenia (6 days later)	260	Normal	
39	2	M	Secondary thrombopenia	20	Hypo	Post merkle
39	2	M	Secondary thrombopenia (21 days later)	350	Normal	
40	3	F	Secondary thrombopenia	20	Hypo	Post merkle
40	3	F	Secondary thrombopenia (9 days later)	250	Normal	
41	2	M	Secondary thrombopenia	20	Hypo	Post merkle
41	2	M	Secondary thrombopenia (28 days later)	250	Normal	
42	5	F	Aplastic anemia	10	Hypo	

\*All platelet counts expressed in thousands

†Counts 24-30 and 38-42 were performed by a method which gives a lower count than Oler's.

‡The platelet samples used in this study were procured after splenectomy. Complete platelet analyses after splenectomy are presented.

marked postoperative increases in the count, gradually returning to normal would indicate that there is an actual hyperplasia of the platelet-forming tissue, thus leading to a still greater release of platelet substances. This would account for the increased skin sensitivity. The skin test upon an idiopathic thrombopenia should be a reliable basis on which to form a preoperative prognosis: the more marked the reaction, the more likely the patient will be to make a good recovery from the purpura after splenectomy. Any patient who appears to belong to this classification and gives a negative reaction should arouse a suspicion of an incorrect diagnosis.

The increased skin sensitivity found in part of the secondary thrombopenias is probably produced by the same mechanism as in the idiopathic type. The secondaries giving a negative reaction may be assumed to have a hypoplasia instead of an excessive destruction. In a hypoplasia or aplasia, where

few platelets are thrown into the blood stream, there will be a lack of disintegration products to stimulate a sensitivity. The fact that both of these secondary groups give a normal reaction after the platelet count has risen supports this theory. The skin test may, therefore, distinguish between excessive destruction and hypoplasia.

The negative reaction of the hemophiliacs is obviously not due to hypoplasia. Another factor is present in this disease which theoretically might lead to less than normal platelet destruction namely the decreased disintegration rate. Since the total count seems to follow the usual normal levels, that is, it is not increased in spite of the lessened disintegration, it is likely that the platelet-producing tissues are not as active as in the normal individuals. The skin sensitivity to platelets may prove to be a useful diagnostic test in hemophilia.

#### SUMMARY

A study of the total and differential platelet counts and disintegration rates, with changes in the differential at the end of the disintegration period was made upon normal men and nonmenstruating women, normal women during menstruation, and hemophiliacs. The majority of the total counts in all groups were between 400 and 600 thousand. Acute colds seemed to cause a definite drop in the count. During menstruation the totals were moderately lowered.

When the count was high in normal individuals the percentage of large platelets in the differential was higher, while in the hemophiliacs the proportion of large platelets was notably decreased. The disintegration rate determinations indicated a 30 per cent destruction is the lower limit of normal. Hemophiliacs and menstruating women gave a relatively retarded rate. The clotting time was delayed up to twenty minutes during menstruation, the normal with the method used being ten to twelve minutes. These points establish an interesting similarity between the blood of hemophiliacs and menstruating women.

Intradermal platelet injections were shown to produce a mild reaction in normal individuals whereas hemophiliacs gave a negative and patients with chronic idiopathic thrombopenic purpuras an increased reaction. One case of aplastic anemia with very few platelets gave a negative reaction. The secondary thrombopenias evidently presented a mixed group some giving an increased reaction, others a negative. All reactions became normal, however, when the platelet count became normal.

The data obtained in this study indicate that platelet differential counts and disintegration rates together with tests of skin sensitivity to platelets, may become important aids in diagnosis and prognosis.

#### REFERENCES

1. Chargaff, E., Bancroft, F. W., and Stanley Brown, M. Studies on the Chemistry of Blood Coagulation. The Chemical Constituents of Blood Platelets and Their Role in Blood Clotting, With Remarks on the Activation of Clotting by Lipids, *J Biol Chem* 116 237, 1936.
2. Olef, I. The Enumeration of Blood Platelets, *J LAB & CLIN MED* 20 410, 1935.
3. Minot, G. R., and Lee, R. I. The Blood Platelets in Hemophilia, *Arch Int Med* 18 474, 1916.

4. Stübel, H.: Ultramikroskopische Studien über Blutgerinnung und Thrombocyten, *Pflüger's Arch. f. d. ges. Physiol.* 156: 361, 1914.
5. Fonio, A.: Über die Gerinnungsfaktoren des Hämophilen Blutes, *Mitt. a. d. Grenzgeb. d. Med. u. Chir.* 28: 313, 1914.
6. Howell, W. H., and Cekada, E. B.: The Cause of the Delayed Clotting in Hemophilic Blood, *Am. J. Physiol.* 78: 500, 1926.
7. Kugelmass, I. N.: The Management of Hemorrhagic Problems in Infancy and Childhood, *J. A. M. A.* 99: 895, 1932.
8. Olef, I.: Rate of Disintegration of Platelets, *J. LAB. & CLIN. MED.* 22: 128, 1936.
9. Erickson, Betty N., Lee, Pearl, Williams, H. H., and Avrin, I.: The Lipid Distribution of Human Platelets in Health and Disease, *J. Clin. Investigation* 18: 81, 1939.
10. Lee, R. I., and Vincent, Beth: Coagulation of Normal Human Blood, *Arch. Int. Med.* 13: 398, 1914.
11. Brakefield, J. L., and Malone, K.: Blood Platelet Numbers in Normal Men and Women, *Ala. M. A. J.* 5: 373, 1936.
12. Genell, S.: Variations in the Number of Blood Platelets During the Menstrual Cycle, *J. Obst. & Gynaec. Brit. Emp.* 43: 1124, 1936.
13. Olef, I.: Differential Platelet Count. Its Clinical Significance, *Arch. Int. Med.* 57: 1163, 1936.
14. Eley, R. E., Green, A. A., and McKhann, C. F.: Use of Blood Coagulant Extract From Human Placenta in Treatment of Hemophilia, *J. Pediat.* 8: 135, 1936.
15. Ferguson, J. H.: Observations on the Alterations of Blood Platelets as a Factor in Coagulation of the Blood, *Am. J. Physiol.* 108: 670, 1934.

## ACTION OF SODIUM THIOCYANATE UPON TISSUE OXYGEN CONSUMPTION\*

DALE G. FRIEND, M.D., BOSTON, MASS., AND  
ROGER W. ROBINSON, M.D., WORCESTER, MASS.

SINCE Pauli<sup>1, 2</sup> described the use of the thiocyanate ion in the treatment of hypertension, this drug has been used as a valuable therapeutic aid in several clinics both in this country and abroad. Favorable reports of the results in lowering blood pressure and relieving cerebral symptoms have appeared frequently in the literature. All investigators have noted toxic effects from the drug in various percentages of their cases. These consist of weakness, nausea, and vomiting, dermatitis, periods of hallucinations, motor aphasia, and even two fatalities with delirium, convulsions, coma, and death. The earlier use of the drug consisted of a daily dose between 1 and 2 gm. of either the sodium or potassium salt. Toxic symptoms were frequent with that dosage. After Barker<sup>3</sup> described the method of determining the thiocyanate concentration in the blood, it was possible to adjust the dose to certain blood concentrations in each patient, and the more serious toxic symptoms from the drug have been experienced less frequently.

With the wide use of this drug, it is surprising that so little is known about its pharmacology and mode of action in lowering blood pressure. Claude Bernard<sup>4</sup> in 1857 described experiments in which he gave large doses of thiocyanate intravenously to dogs and came to the conclusion that the drug was a direct smooth muscle poison. It abolished muscular activity without

\*From the Department of Biochemistry of the Harvard Medical School and the Medical Clinics of the Peter Bent Brigham Hospital, Boston.  
Received for publication, October 22, 1938.

producing any sensory change Paul<sup>1 2</sup> and Bentley and LeRoy<sup>5</sup> thought that the beneficial results in arteriosclerosis were due to the power of thioeyanate to dissolve calcareous deposits in the arteriosclerotic areas in the arteries Logesfiel<sup>6</sup> opposed this viewpoint because of the prompt drop in blood pressure produced by the drug and the fact that the drop was not permanent Paul<sup>1 2</sup> also suggested that the fall in blood pressure might be due to a "decrease in the energy of the heart muscle" In this regard Palmer, Silver, and White<sup>7</sup> noticed a softening in the force of the heartbeat in some hypertensive cases receiving thioeyanate Nichols<sup>8</sup> believes that in toxic doses, thioeyanate is a direct muscle poison abolishing muscle activity It is known that the thioeyanate ion diffuses equally into all extracellular fluids with the same ease as urea Because of this property, it has been used by Crandall and Anderson<sup>9</sup> as a method of determining the total amount of extracellular fluid Takaes<sup>10</sup> believed that thioeyanate was a powerful vagal stimulant and explained the drop in blood pressure on this basis Westphal and Blum<sup>11</sup> suggested that thioeyanate "increases the permeability of the arterioles and decreases their tonus"

From this discussion it is evident that several theories have been advanced for the action of thioeyanate, but none is substantiated by sufficient evidence to warrant its acceptance One of us in studying a large group of cases with essential hypertension with this drug was impressed by the frequent statement by patients that they were weak, tired, lazy or lacked energy Some volunteered the information that their skin was unusually dry and they noticed cold weather more than previously They exhibited symptoms suggestive of depression of their metabolism

With these observations in mind we studied the action of sodium thioeyanate upon the oxygen consumption of normal rat liver in human serum Blood was drawn from hypertensive patients receiving no medication, and the serum was prepared free of carbon dioxide as described elsewhere<sup>1</sup> A portion of the serum was used for control experiments without thioeyanate Sodium thioeyanate in varying amounts was added to the remaining portion The  $QO_2$  was determined according to our previously described technique<sup>1</sup>

From Table I it can readily be seen that the drug does depress the oxygen consumption of normal tissue Of course, the higher concentrations that were studied are far above the therapeutic and well into the toxic range The lower levels, however, are occasionally seen in patients

The limiting concentrations the effect of which can be detected with this procedure, appear to be between 20 and 30 mg per 100 cc

It is interesting to point out that sodium thioeyanate depresses the oxygen consumption of rat liver almost as much as does a similar dose of chloral hydrate<sup>2</sup> The liver tissue so depressed by thioeyanate, we have found from some preliminary experiments, is capable of being stimulated by methylthioumine chloride when it is suspended in human serum

We next studied the action of serum from patients receiving sodium thioeyanate upon oxygen consumption of rat liver The tissue, suspended in the serum, was compared with controls suspended in serum from another



TABLE I

ACTION OF SODIUM THIOCYANATE ON OXYGEN CONSUMPTION OF RAT LIVER

NUMBER	MG. NaSCN PER C.C.	QO <sub>2</sub> NO NaSCN	QO <sub>2</sub> WITH NaSCN	% REDUCTION OF O <sub>2</sub> CONSUMPTION
1	8.0	-14.7	- 9.2	-37
2	4.0	-14.7	-10.5	-28
3	2.0	-11.3	- 9.4	-17
4	0.3	-14.0	-11.9	-15
5	0.2	-12.1	-10.8	-11
6	0.15	-12.1	-11.6	- 5
7	0.15	-10.2	-10.2	- 0

hypertensive patient who was not receiving sodium thiocyanate. The sodium thiocyanate of the serum was checked by chemical analysis. The drug content varied from 8 to 22 mg. per 100 c.c. These amounts fall in the lower limit of our method, thus making it doubtful whether we would be able to detect the effect of the presence of the drug in serum containing less than 20 mg. per 100 c.c. (see Table II).

These results, although indicating that there is some decreased oxygen consumption, are not striking, in view of the fact that variations in the oxygen consumption of less than 10 per cent are probably of questionable significance. We are at present unable to say whether there is a definite decreased oxygen consumption at the therapeutic levels or not. Nevertheless, in concentration

TABLE II

ACTION OF SODIUM THIOCYANATE SERUM ON OXYGEN CONSUMPTION OF RAT LIVER

PATIENT	BLOOD NaSCN MG. %	QO <sub>2</sub> HYPERTENSIVE CONTROL SERUM	QO <sub>2</sub> HYPERTENSIVE NaSCN SERUM	% DECREASE IN O <sub>2</sub> UPTAKE
1	10.6	-13.6	-12.3	- 9.8
2	12.1	-10.2	- 9.7	- 4.9
3	10.3	-12.7	-12.4	- 2.4
4	9.5	-11.3	-10.6	- 6.2
5	8.0	-12.1	-11.8	- 2.5
6	9.6	- 9.7	-10.0	- 3.1
7	22.0	-10.0	- 8.5	-13.0

ranges where the action of this drug can be studied using our technique, it apparently exerts a definite depressing effect upon tissue respiration. It should be remembered that in our experiments the tissue is in contact with the serum plus thiocyanate only one hour, while the cells in the patient are constantly exposed to it for weeks. This depressing effect is analogous to the effect of traces of potassium cyanide and chloral hydrate. Just where in the oxidation chain thiocyanate exerts its influence we are unable to say. It does not interfere with the stimulating action of methylthionine chloride. However, one patient who exhibited toxic symptoms from the drug was not relieved when methylthionine chloride was given orally for forty-eight hours.

## SUMMARY

1. Oxygen consumption of rat liver cells is depressed by the thiocyanate ion when added to serum in vitro.

2 Serums from patients receiving thiocyanate and exhibiting concentrations of the drug from 8 to 22 mg per 100 cc do not appear to depress the oxygen consumption of rat liver suspended in them

3 Methylthiourea chloride, when given orally, does not exert any anesthetic effect

## REFERENCES

- 1 Pauli, W Munchen med Wchnschr 50 155 1903
- 2 Pauli, W Physical Chemistry in the Service of Medicine Translated by M H Fisher, New York, 1906, Wiley & Sons
- 3 Barler, M H Blood Cyanates in the Treatment of Hypertension, J A M A 106 762, 1936
- 4 Bernard, C Lecons sur les effets des substances toxiques et medicamenteuses 7 1857
- 5 Bentley, W , and LeRoy, B R The Solvent Action of the Sulfocyanates Upon the Lime and Magnesium Deposits in the System New York M J 88 210 1908
- 6 Logefiel, R C Observations on the Use of Potassium Sulfocyanate in the Treatment of Essential Hypertension, Minnesota Med 12 151 1929
- 7 Palmer, R S, Silver, L S, and White, P D Clinical Use of Potassium Sulfocyanate in Hypertension, New England J Med 201 700 1929
- 8 Nichols, J B Pharmacologic and Therapeutic Properties of Sulfocyanate, Am J M Sc 170 735, 1925
- 9 Crandall, L A, and Anderson, M A Estimations of the State of Hydration of the Body by Amount of Water Available for Solution of Sodium Thiocyanate, Am J Digest Dis & Nutrition 1 126, 1934
- 10 Takacs, L Thiocyanates, Ztschr f d ges exper Med 50 432 1926
- 11 Westphal, K, and Blum, R Die Rhodanthérapie des genuineu Arteriellen Hochdrucks und ihre theoretische Begründung Deutsches Arch f klin Med 152 331, 1926

# LIVER DYSFUNCTION AS A POSSIBLE CAUSATIVE FACTOR IN RENAL LITHIASIS\*

## A CLINICAL INVESTIGATION ON 39 PATIENTS

WILLIAM J. EZICKSON, A.B., M.D., PHILADELPHIA, PA.

### INTRODUCTION

THESE clinical investigations concerning the possible role of the liver in the pathogenesis of renal lithiasis, we believe, are the first to have been undertaken. During the past ten months, studies have been made in the Calculus Research Clinic at the Pennsylvania Hospital, Philadelphia. Liver function tests were made on individuals who have or have had renal or ureteral calculi. We believe the findings are intensely interesting and warrant bringing them to your attention. It is possible this may open up a new avenue to the approach of the problem of urinary lithiasis.

*Previous Studies and Findings That Lead to This Investigation.*—The author,<sup>1</sup> in collaboration with Dr. J. B. Feldman, presented before the American Medical Association, at Atlantic City, in June, 1937, a paper which dealt with the incidence of vitamin A deficiency in a group of individuals with urinary lithiasis. It was shown in this study that 96 per cent of these patients showed a vitamin A deficiency. It was also shown that the giving of vitamin A concentrate by mouth in large amounts over a period of six to nine months did not alter this deficiency. This suggested to us the possibility that in individuals with urinary lithiasis, there was either a lack of assimilation of vitamin A, or if assimilation did take place, a lack of utilization due to some unknown disturbance of metabolism. Since the liver is known to be the storehouse for this vitamin, we decided to turn our attention to the liver and determine, if possible, if there was any disturbance in the liver function of these patients.

*Liver Function Test Used in These Studies.*—We used the bromsulphalein test in making these studies. We believe this test to be as reliable as any, and in a recent article on the use and interpretation of tests for liver function, Snell and Magath<sup>2</sup> state: "In types of disease of the liver not associated with jaundice, information gained from the study of retention of bromsulphalein is as reliable as that which can be gained in any other way, and that under these conditions, other tests give chiefly confirmatory evidence."

In most of the cases we injected 5 mg. of the dye per kilogram of body weight, as suggested by O'Leary, Greene, and Rowntree.<sup>3</sup> In a few cases we

\*From the Calculus Research Clinic, Department of Urology, Pennsylvania Hospital, Philadelphia.

Presented before a combined meeting of the Philadelphia Urological Society and the New York Urological Society, Philadelphia, April 21, 1938.

Received for publication, October 25, 1938.

injected 2 mg of the dye per kilogram of body weight. This latter dosage was proposed at first, but later it was shown that the test could be improved by injection of the larger dose (5 mg per kg of body weight).

*Analysis of Results of Bromsulphalein Test*—Group 1 was given 5 mg per kg nonfasting, and a thirty minute specimen was taken. In this group, which consisted of 30 cases, 15 showed dye retention of over 10 per cent, and of these 15, 9 had retention of 14 per cent to 20 per cent, 4 had 20 per cent to 50 per cent, and 2 had over 50 per cent. Table I shows these results in detail.

TABLE I

## BROMSULPHALEIN RETENTION

Group 1    5 mg per kg of the Dye Injected, 30 Minute Specimen, Nonfasting

CASE	PER CENT	CASE	PER CENT
1	42	16	18
2	15	17	10
3	4	18	16
4	3	19	43
5	19	20	8
6	21	21	10
7	0	22	10
8	6	23	Traces
9	9	24	18
10	Traces	25	2
11	14	26	22
12	16	27	4
13	Traces	28	16
14	16	29	58
15	60	30	7

Group 2 was given 5 mg per kg on a fasting stomach and a thirty minute specimen was taken. In this group which consisted of 10 cases, 9 had retention of the dye over 10 per cent of which 5 had retention of 13 per cent to 20 per cent and 4 had 20 per cent to 40 per cent. Table II shows these results in detail.

TABLE II

## BROMSULPHALEIN RETENTION

Group 2    5 mg per kg of the Dye Injected, 30 Minute Specimen, Fasting

CASE	PER CENT	CASE	PER CENT
2	26	32	16
17	13	33	40
19	1	34	13
24	17	35	32
31	14	39	38

Group 3 was given 2 mg per kg, on a fasting stomach and a thirty minute specimen was taken. In this group, which consisted of 7 cases, 3 had retention of 3 per cent to 5 per cent, and one was over 20 per cent. Table III shows these results in detail.

Certain cases appear in more than one group, and can be followed by the particular case numbers, which would be the same in each group. This was done to check one method of giving the dye against another, and the results obtained.

TABLE III

## BROMSULPHALEIN RETENTION

Group 3. 2 mg. per kg. of the Dye Injected, 30-Minute Specimen, Fasting

CASE	PER CENT	CASE	PER CENT
1	5	36	1
12	27	37	3
26	1	38	3
29	1		

TABLE IV

CASE	AGE	SEX	CALCULUS AT PRESENT	LOCATION	PREVIOUS OPERATIONS	CALCULUS PASSED SPONTANEOUSLY
1	46	M	Yes	Lt. kidney	1932 Rt. ureterolithotomy	No
2	49	F	Yes	Rt. kidney	1925 Rt. pyelolithotomy 1931 Rt. pyelolithotomy 1935 Lt. nephrectomy	No
3	34	F	Yes	Rt. kidney	1936 Rt. ureterolithotomy	No
4	22	F	Yes	Rt. ureter	None	Yes
5	44	M	No		None	Yes
6	49	F	No		1932 Rt. nephrectomy	No
7	34	M	No		1934 Rt. nephrectomy	No
8	33	M	Yes	Lt. kidney	1922 Bladder stone-cystotomy 1925 Rt. nephrectomy 1932 (Sept.) Lt. ureterolithotomy 1932 (Oct.) Lt. ureterolithotomy	Yes
9	61	F	Yes	Lt. ureter	1932 Rt. ureterolithotomy	No
10	14	M	No		1937 Rt. nephrectomy	No
11	55	M	No		1937 Lt. nephrectomy	No
12	48	F	No		1934 Lt. nephrectomy	No
13	35	M	Yes	Rt. kidney pelvis	None	No
14	28	M	Yes	Lt. kidney	1925 Lt. pyelolithotomy 1934 Rt. nephrectomy	Yes
15	41	M	No		None	Yes
16	36	M	Yes	Lt. kidney	1938 Lt. nephrolithotomy	Yes
17	27	M	Yes	Both kidneys	1936 Rt. ureterolithotomy	Yes
18	37	F	Yes	Lt. ureter	None	No
19	27	F	No		1936 Rt. ureterolithotomy	Yes
20	39	F	No		1937 Lt. pyelolithotomy	No
21	41	M	No		1936 Lt. nephrectomy	No
22	32	F	No		1927 Rt. pyelolithotomy 1937 Rt. pyelolithotomy	No
23	41	M	Yes	Rt. ureter	None	No
24	45	F	Yes	Both kidneys	1933 Lt. pyelolithotomy 1935 Rt. ureterolithotomy	No
25	42	M	Yes	Rt. kidney	1936 Rt. nephrectomy 1936 Lt. pyelolithotomy	Yes
26	48	F	Yes	Lt. kidney	None	No
27	40	F	No		1937 Rt. ureterolithotomy	No
28	31	M	No		1937 Lt. nephrolithotomy	Yes
29	42	M	Yes	Lt. kidney	None	No
30	36	F	No		1937 Rt. ureterolithotomy	No
31	28	F	No		1938 Lt. nephrectomy	Yes
32	46	M	No		1925 Lt. pyelolithotomy	Yes
33	47	F	No		1928 Lt. pyelolithotomy	No
34	42	M	Yes	Lt. ureter	None	Yes
35	42	F	No		None	Yes
36	20	F	Yes	Lt. kidney	None	No
37	20	F	Yes	Rt. ureter	None	Yes
38	41	M	Yes	Lt. kidney	None	No
39	39	F	Yes	In stump of rt. ureter	1930 Rt. pyelolithotomy 1931 Rt. nephrectomy (subcapsular)	No

*It is most important to bear in mind that none of these patients had any clinical evidence of liver disease.* Under these circumstances we believe the figures, showing such a large percentage of these patients having a high percentage of dye retention, to be rather significant.

*Data Pertaining to the History of Calculi in These Patients.*—All of the individuals studied in this investigation have or have had renal or ureteral calculi. The details concerning their calculi history are shown in Table IV. This table shows (1) age, (2) sex, (3) whether or not the patient has calculus at the present time, and (4) if present, its location. (5) previous operations renal or ureteral, if any, (6) whether or not calculus passed spontaneously. An analysis of these patients shows the following: Average age 38 plus, 19 males, 20 females, 21 have calculi at the present time located as follows: 4 in the right kidney, 7 in the left kidney, 2 bilateral renal calculi, 4 in the right ureter, 3 in the left ureter, 1 in the left kidney and right ureter.

Twenty six have had renal or ureteral surgery which included: 4 pyelolithotomies, one nephrolithotomy, 7 ureterolithotomies, 7 nephrectomies, 4 pyelolithotomies and nephrectomies, one had 2 pyelolithotomies, one had 2 pyelolithotomies and a nephrectomy, one had a cystotomy, 2 ureterolithotomies and a nephrectomy. Fifteen have passed calculi spontaneously.

The case numbers in this table correspond to the same case numbers shown in Tables I, II, and III.

*Studies for Vitamin A Deficiency.*—Of this group of 39 patients, 34 were studied for vitamin A deficiency. Thirty two were found deficient and 2 were normal. The studies for vitamin A deficiency were made by means of the dark adaptation test, using the Feldman Adaptometer. The method and instrument have been described in recent literature.<sup>4,6</sup> We used this method as it is held to be the most reliable and also the most scientific. Yndkin<sup>7</sup> in a recent article, states that this method is the most satisfactory.

The fact that 94 per cent of the tested group showed vitamin A deficiency is very significant when compared to the observations made by Moore and Ellison.<sup>8</sup> They analyzed the amount of vitamin A concentration in the livers of 1000 adults that came to autopsy. The lowest concentrations were found in those patients who died of kidney and bladder infections.

#### SUMMARY

1 Studies of the liver function were made on 39 individuals who have or have had renal or ureteral calculi.

2 The bromsulphalein test was used to determine the liver function.

3 In group 1 (given 5 mg per kg, thirty minute specimen, nonfasting) 50 per cent showed retention of the dye, ranging from 14 per cent to 60 per cent.

In group 2 (given 5 mg per kg, thirty minute specimen, fasting) 90 per cent showed retention of the dye, ranging from 13 per cent to 40 per cent.

4. Thirty-four patients in this group were studied for vitamin A deficiency and 32 (94 per cent) showed a deficiency.

5. The findings in this investigation lead us to believe that there is a close relationship between liver dysfunction, vitamin A deficiency, and renal lithiasis. This should open up a new avenue to the approach of the problem of the pathogenesis of renal calculi, and we believe this should stimulate further investigations along this line.

I wish to thank Dr. John T. Bauer, Director of the Clinical Laboratory of the Northwestern University Medical School, and his associate, Dr. William Wolff, for their cooperation in making these studies.

#### REFERENCES

1. Ezickson, William J., and Feldman, J. B.: Signs of Vitamin A Deficiency in the Eye Correlated With Urinary Lithiasis, *J. A. M. A.* 109: 1706, 1937.
2. Snell, Albert M., and Magath, Thomas B.: The Use and Interpretation of Tests for Liver Function, *J. A. M. A.* 110: 167, 1938.
3. O'Leary, P. A., Greene, C. H., and Rowntree, L. G.: Diseases of the Liver. VIII. The Various Types of Syphilis of the Liver With Reference to Tests for Hepatic Function, *Arch. Int. Med.* 44: 155, 1929.
4. Feldman, J. B.: Dark Adaptation as a Clinical Test, *Arch. Ophth.* 15: 1004, 1936.
5. Feldman, J. B.: Dark Adaptation as a Clinical Test. Further Studies, *Arch. Ophth.* 17: 648, 1937.
6. Feldman, J. B.: Instrument for the Qualitative Study of Dark Adaptation, *Arch. Ophth.* 18: 821, 1937.
7. Yudkin, A. M.: Vitamins in Treatment and Prevention of Ocular Diseases, *Arch. Ophth.* 19: 365, 1937.
8. Current Comment: Vitamin A Reserves in Health and Disease, *J. A. M. A.* 109: 590, 1937.

## ELECTROCARDIOGRAPHIC CHANGES FOLLOWING THE INTRAVENOUS ADMINISTRATION OF MAGNESIUM SULFATE\*

### II. AN EXPERIMENTAL STUDY ON DOGS

T. R. VAN DELLEN, B.S., M.D., AND J. ROSCOE MILLER, M.S., M.D.  
CHICAGO, ILL.

**I**N A previous study<sup>1</sup> it was shown by means of electrocardiographic studies that magnesium, when injected intravenously, has a definite effect upon the cardiac conduction system. This effect consisted of an early acceleration followed by slowing, with delay in the auriculoventricular and ventricular conduction time and increased excursion of all complexes. This effect is not permanent.

Continued study seemed indicated to determine whether this was a direct effect upon the conductive system and what part, if any, the vagus nerve had in the result.

Di Macco<sup>2</sup> was of the opinion that the action of magnesium on cardiac activity was dependent on vagal effect. His conclusions were based on blood pressure readings. He further observed that the effect was most pronounced when atropine was given after magnesium had been injected.

\*From the Department of Medicine, Northwestern University Medical School.  
Received for publication, November 4, 1938.

## METHODS

As in previous experiments<sup>1</sup> the unanesthetized dog was placed on the left side and electrodes attached to the right front and left rear legs. Electrocardiograms were made on a string galvanometer type machine. Both magnesium and atropine were injected into the right saphenous vein.

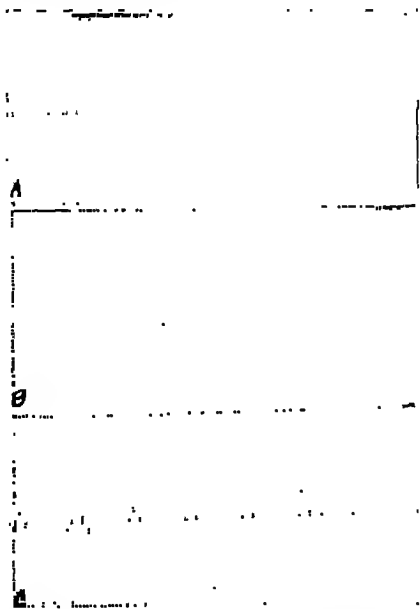


Fig. 1.—Dog B (14 kg.). A, Normal graph. B, After injection of atropine sulfate. C, After injection of 10 c.c. of a 20 per cent solution of magnesium sulfate.

## RESULTS

*Experiment 1.*—In an attempt to determine the effect of atropine alone, 3 dogs were injected with small, so-called stimulating doses, 0.01 mg. per kg., and then large paralyzing doses, 0.2 mg. per kg.<sup>3</sup> Electrocardiograms were made during injection and at intervals for three hours afterward. The results were similar to those described by Halsey,<sup>4</sup> the changes in rate being the most significant.

*Experiment 2.*—Three dogs were given a large dose of atropine sulfate (0.2 mg. per kg. body weight). The solution was of such concentration that 1 c.c. contained 0.4 mg. of atropine. Immediately following injection, one dog was given 10 c.c. of a 20 per cent solution of magnesium sulfate, while the other two were given 20 c.c. of the same solution. The magnesium sulfate in these experiments was injected intravenously at the rate of 2 c.c. per minute.



The effect was the same as that seen when magnesium sulfate was given without a preceding injection of atropine (Fig. 1). The rate was slower first, but later accelerated. The P-R interval increased from 0.09 to 0.12 second, and the QRS complex from 0.04 to 0.06 second. The height and contour of the P- and T-waves were changed in the same manner as in the unatropinized animals.

All of these results became more pronounced with the increased dose of magnesium.

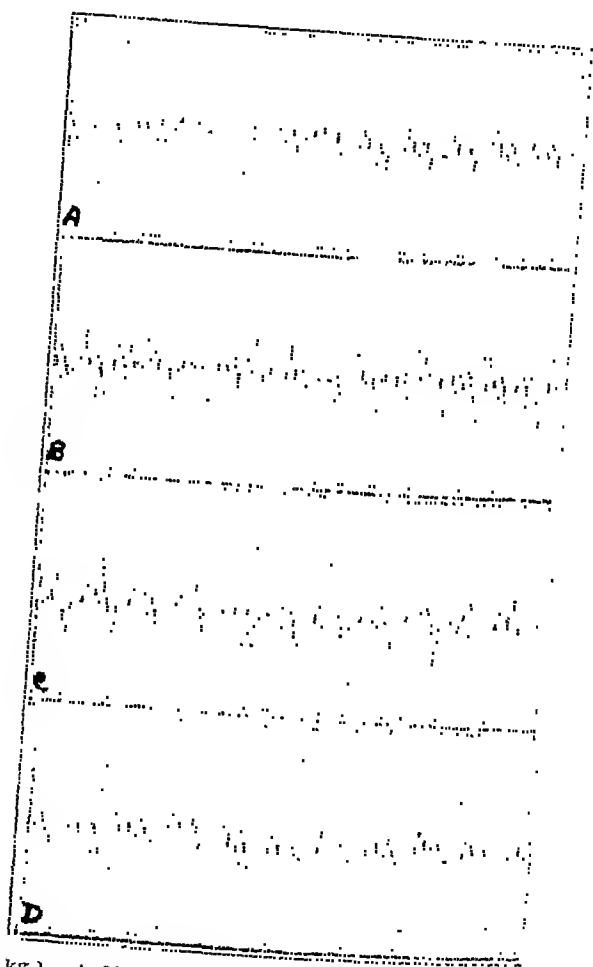


Fig. 2.—Dog V (10.7 kg.). *A*, Normal graph. *B*, After bilateral vagotomy. *C*, After injection of magnesium sulfate solution. *D*, After injection of atropine sulfate.

*Experiment 3.*—One animal was anesthetized and the vagi were sectioned. Four hours later, when recovery was apparently complete, an electrocardiogram showed no change when compared with one made previous to operation, except that the rate was increased. Ten cubic centimeters of a 20 per cent solution of magnesium sulfate was then injected intravenously and records made during and after the injection.

The result (Fig. 2) corresponded to that obtained in animals with unsectioned vagi. The P-R interval was increased from 0.09 to 0.14 second, and

the QRS complex from 0.04 to 0.08 second. A large dose of atropine, 0.2 per kg, was then given, with no demonstrable results.

# SUMMARY

These experiments indicate that the effect of magnesium upon the cardiac conductive system is not central but direct.

# REFERENCES

1. Miller, J. Roscoe, and Van Dellen, T. R.: Electrocardiographic Changes Following the Intravenous Administration of Magnesium Sulfate. An Experimental Study on Dogs, J. LAB. & CLIN. MED. 23: 914, 1938.
2. Di Maccio, G.: The Effect of Atropine on the Hypotensive Action of Magnesium, Riv. di pat. sper. 5: 393, 1930.
3. Pelcher, J. D., and Sollmann, Torald.: Quantitative Studies of Vagus Stimulation and Atropine, J. Pharmacol. 5: 317, 1914.
4. Halsey, J. T.: The Digitalized Dog's Heart as Affected by Amyl Nitrate or Atropine Studied Electrocardiographically, J. Exper. Med. 25: 729, 1917.

# LABORATORY METHODS

## SODIUM FERROCYANIDE EXCRETION IN THE ESTIMATION OF RENAL FUNCTION\*

MILTON PLOTZ, M.D., F.A.C.P., AND MARGARETHE ROTHENBERGER, M.D.  
BROOKLYN, N. Y.

WITH THE TECHNICAL ASSISTANCE OF EDGAR FERGUSON, AND  
VICTOR GINSBURG, M.D.

THE use of sodium ferrocyanide in estimating renal function was first proposed by Stieglitz and Knight<sup>1</sup> in 1934. The present study was undertaken on the wards of the Long Island College Division of the Kings County Hospital as part of a project for estimating the clinical value of kidney function tests. Two-hour phenolsulfonphthalein, fractional phenolsulfonphthalein, concentration tests, urea clearances, detailed analyses of the blood, urinary and clinical data were done in combinations of three or more of these on each patient and in most cases all the tests were performed. This report will be concerned only with the results obtained with sodium ferrocyanide; other details of the project will be reported in detail elsewhere.

Stieglitz and Knight,<sup>1</sup> on the basis of experimental work done on the rabbit, proposed that their test be considered essentially as a measure of pure glomerular filtration. Van Slyke, Hiller, and Miller<sup>2</sup> found additional evidence to support the view of Gersh and Stieglitz<sup>3</sup> that, in the dog and rabbit at least, sodium ferrocyanide excretion is a true measure of glomerular filtration. That these conclusions cannot be carried over to man is indicated by Miller and Winkler,<sup>4</sup> whose data suggest that, in contrast to the situation in the dog, 40 per cent of the ferrocyanide excreted by the human glomerulus is reabsorbed by the tubule. Of course, this finding does not mean that sodium ferrocyanide is without value as a renal function test; it means only that, in any case, it cannot be accepted only as a test of glomerular function.

*Method.*—The method of Stieglitz and Knight<sup>1</sup> was followed in detail but was occasionally supplemented by a modification in titration in order to get added accuracy in determining the end point. The drug, generously supplied by the Abbott laboratories in ampoules containing 0.25 gm. of the anhydrous

\*From Kings County Hospital, Long Island College Division, the medical services of Dr. Tasker Howard and Dr. Carl H. Greene.

This study was made possible by a generous grant from the Harriet Weinbaum Foundation.

Clinical studies in renal function No. 4 from the Long Island College of Medicine.  
Received for publication, July 29, 1938.

salt, was dissolved in 10 cc of sterile distilled water. In some cases, in order to compare the urea clearance and the ferrocyanide test, the two were run simultaneously. The urine was collected at fifteen, thirty, sixty, and one hundred twenty minutes. It was found that urine passed during the third hour usually contained very little ferrocyanide, and the collection of a one hundred eighty minute specimen was abandoned. The titration was done twice, first, with 0.4 per cent copper sulfate, as suggested by Stieglitz and Knight, and again, as a check, with 0.1 per cent copper sulfate. The latter modification was suggested by the finding that, when using a small sample, it is difficult to get an accurate reading with the amount of 0.4 per cent copper sulfate used. A quicker and simpler method of titration was used as a supplement in some of the later cases. Standards were set up containing the maximum and minimum expected percentages of ferrocyanide (diluting from a weighed standard) in about ten steps. To 10 cc of these standard solutions was added 1 cc of 0.5 per cent ferric chloride. This was repeated with the unknown. These standards will keep for several days, the color comparison is easily made and corrected for turbidity as in the standard phenolsulfonphthalein procedure.

### RESULTS

**A Normals**—Seventy eight young adults on the medical wards were carefully selected for their freedom from any signs of kidney insufficiency. Most of them were suffering from peptic ulcer or arthritis although there was an occasional drug addict or diabetic. In 17 cases, the ferrocyanide test indicated markedly diminished renal function. In all of these patients excretion of phenolsulfonphthalein, urea clearance, and urine concentration tests were perfectly normal. In other words, there was a 22 per cent error with ferrocyanide. In the same series there was a 14 per cent error with the two hour phenol sulfonphthalein test. In only one case did both the phenolsulfonphthalein and the ferrocyanide excretions indicate diminished renal function in the same patient. In this case, a repetition of the phenolsulfonphthalein test gave normal figures, thus was not true for ferrocyanide. The thirty minute phenolsulfonphthalein test was in error in only 8 per cent of these normals and, in all but one case, normal figures were obtained when the test was repeated. At this point it should be emphasized that every test for kidney function now in common clinical use may occasionally give an erroneous picture in normal patients and later show normal function when repeated under somewhat different conditions or at another time of the day. It is well to bear this point in mind before concluding, on the evidence of a single unrepeatable test, that a patient is suffering from impaired renal function.

**B Known Kidney Disease**—There were 19 patients with known kidney disease in whom the urea clearance was definitely diminished and in whom there was unmistakable clinical evidence of nephritis or nephrosclerosis. In two of these there was normal ferrocyanide excretion even when the test was repeated. In one patient there was a subacute hemorrhagic nephritis with a blood pressure at rest of 150/95, hematuria, and a low urea clearance. The excretion of ferrocyanide and phenolsulfonphthalein (both thirty minutes and two hours)

were within normal limits. In the second patient, one of advanced nephrosclerosis, urine concentration tests, urea clearance, phenolsulfonphthalein, and blood urea agreed, but there was normal ferrocyanide excretion. In this case it appears that the ferrocyanide test gave a definitely erroneous impression; the first case is more difficult to interpret. On the whole, we felt that the urea clearance gave the most accurate picture of the case. The subsequent course of the patient could not be followed, so it is impossible to state whether or not she went on to more advanced impairment of renal function.

*C. Hypertension.*—Stieglitz and Knight analyzed a series of 11 cases of hypertension, with adequate cardiac efficiency and no urinary evidence of kidney injury. They found that the ferrocyanide excretion was diminished more often and more markedly than that of phenolsulfonphthalein. This they took as evidence of the greater sensitivity of the ferrocyanide test to changes in the glomerular circulation, which was to be expected in view of the predominantly glomerular excretion of ferrocyanide and the tubular excretion of phenolsulfonphthalein. We investigated a series of 27 similar cases. Fifteen had normal renal function tests; the ferrocyanide excretion was below normal in 12, being considerably reduced in 3. Phenolsulfonphthalein was reduced to a much smaller degree and in only 7 cases, all of which had lowered ferrocyanide excretion. The urea clearance was diminished in only 8 of the 27 patients. These results, although not based on enough cases to draw any conclusions, would seem to confirm the opinion of Stieglitz and Knight that ferrocyanide excretion is diminished earlier in hypertension than other tests of renal function. One should not, on the basis of these results, hasten to assume without further evidence that glomerular inefficiency is the cause of the hypertension in the cases which show renal function impairment.

*D. Heart Failure.*—There were 21 cases of cardiac decompensation. Fifteen showed moderate to severe renal involvement, as shown by the ferrocyanide test. All of these showed improvement as the cardiac failure improved.

*Reactions.*—There were 251 injections in 145 patients. Fourteen times there were reactions, 6 per cent of all injections, 9.6 per cent of all patients. Where there was a reaction, the injection was never repeated. Where there was no reaction to the first injection, there was no reaction to subsequent ones. In 7 patients there was nausea or vomiting; in 6 there were weakness and dizziness or syncope; one had an anuria lasting eight hours. The most severe reaction occurred in the normal control group. The patient developed a gross hematuria which continued for three hours, after which the urine was normal in every respect.

#### CONCLUSIONS

1. The sodium ferrocyanide test was performed 251 times on 145 patients.
2. It was not so reliable as the fractional phenolsulfonphthalein test or the urea clearance test in revealing normal renal function in a control group.
3. It was slightly less reliable in revealing impairment of kidney function in renal disease.
4. It was somewhat more sensitive in detecting early renal damage in hypertension than other function tests.

5 Reactions occurred in 96 per cent of cases. None were fatal or permanent.

6 For routine use, the test has no advantage over the fractional phenol sulfonphthalein or urea clearance.

#### REFERENCES

- 1 Stieglitz, E. J. and Knight, A. A. Sodium Ferrocyanide as a Clinical Test of Glomerular Efficiency, Preliminary Report, J. A. M. A. 103 1710, 1934
- 2 Van Slyke, D. D., Hiller, A., and Miller, B. I. The Clearance, Extraction Percentage and Estimated Filtration of Sodium Ferrocyanide in the Mammalian Kidney. Comparison With Inulin, Creatinin and Urea, Am. J. Physiol. 113 611, 1935
- 3 Gersh, I., and Stieglitz, E. J. Histochemical Studies in the Mammalian Kidney. I. The Glomerular Filtration of Ferrocyanide in the Rabbit and Some Related Problems, Anat. Rec. 58 349, 1934
- 4 Miller, B. F., and Winkler, A. Ferrocyanide Excretion in Man. J. Clin. Investigation 15 489, 1936

### A DEVICE FOR VIEWING PRECIPITIN REACTIONS\*

ELIZABETH M. KANE, M. A. and JANET R. McCARTER, Ph. D.  
MADISON, WIS.

**P**RECIPITIN reactions have usually been read against a window but with sunlight illumination, or against a background of black cloth with an electric light. Under these conditions readings by the same individual on the same antigen-antibody system have varied considerably with the intensity of the sunlight, and even under apparently similar conditions readings by two persons have varied slightly. Readings for rings of turbidity have been consistent when made with the use of the lighting box designed for us by Dr. W. R. Kane, and illustrated in Fig. 1. Although other devices<sup>1</sup> have been suggested, this one possesses features of simplicity in both construction and equipment. It has also been found satisfactory for reading Kahn tests.

The principal feature of the box is that no direct light from the source of illumination can reach the eye. The angle at which the tubes are viewed is defined by two horizontal slits such that the background is a strip of black velvet just below the ground glass diffusing screen. The scattered light from the precipitate is maximum, since the angle of scattering is small. Direct reflections are avoided by painting the slit and neck flat black (no luscious oil or varnish) and hiding the bottoms of the tubes below the second slit. This permits the tests to be read with very little eyestrain. Constant conditions are obtained by being entirely independent of daylight and by placing the

\*From the Department of Agricultural Bacteriology, College of Agriculture, University of Wisconsin.

This work was supported in part by a grant from the Wisconsin Alumni Research Foundation and is published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Received for publication June 27, 1938

tubes at some standard distance from the eye and from the light. A 60 watt bulb appears to provide sufficient illumination, although the box is wide enough (10 inches) to take a 200 watt lamp.

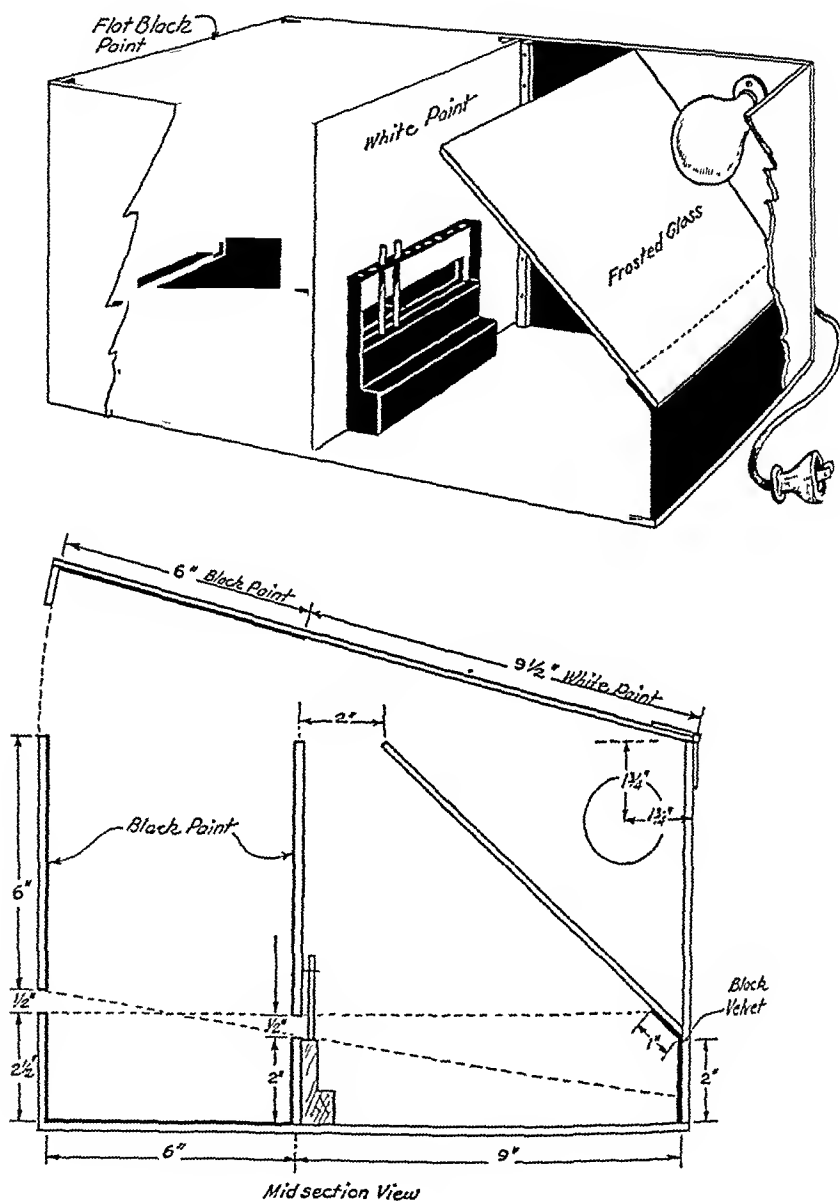


Fig. 1.

A smooth bed on which the tube rack could be slid into position from the side instead of lowering it from the top would improve the apparatus. Larger racks and partitions with slits enlarged downward would permit it to be used also for agglutination and hemolysin tests.

#### REFERENCE

1. Nickolls, L. C., and Thomas, John C.: An Improved Precipitation Viewing Apparatus. Brit. J. Exper. Path. 18: 55, 1937.

## A NEW BLOOD CULTURE MEDIA APPARATUS\*

PETER NARVAEZ, ELDREDGE, CALIF

THE success of making blood culture media is dependent directly upon the sterility that can be maintained during the transfer of blood to the media. Very often during this transfer the media become contaminated, since the blood must be added below 50° C. This article and diagram describe a new

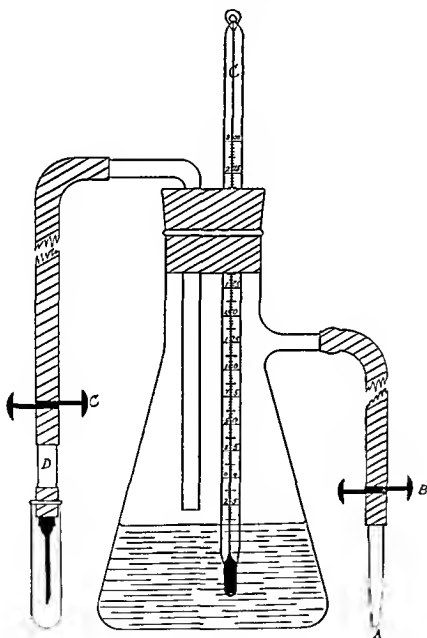


Fig. 1

method which eliminates contamination to a great extent. It is inexpensive, easily constructed, and very well suited to a small laboratory. This method has been used in the laboratory of the Sonoma State Home for the past two years with less than 1 per cent contaminations. The principle is here outlined

\*From the Bacteriological Laboratory, Sonoma State Home, Eldridge.  
Received for publication July 27, 1938.



Into a 1,000 c.c. suction flask, place 500 c.c. of bullion together with agar, stopper with a two-hole rubber stopper. In one hole place a 300° C. thermometer; in the second hole place a glass tube bent at right angles so that it reaches 1 inch above the media. The other end has a rubber tube 8 inches long fitted with a No. 9 gauge needle. Glass tube *D* can be placed somewhere near needle. Clamp rubber tube. Place a small test tube over needle to insure sterility at all times.

On the suction tip place a rubber tube 4 inches long, with a glass tube protruding and clamp in the middle. The entire apparatus, together with the media, is now ready to be autoclaved.

After the media have cooled to a proper degree, a Bunsen burner is held over glass tip *A*, and clamp *B* is opened to admit sterile air, after which the blood is to be added.

Remove test tube covering sterile needle and make vein or heart puncture. When clamp *C* is released, the blood enters the flask and is mixed with the media. When a suitable mixture is obtained, clamp as before and remove rubber tube from suction tip. From this tip, tubing or the pouring of plates can be accomplished very successfully with the media being sterile at all times. To insure greater sterility, an alcohol lamp can be held over glass tube *D* during the pouring of media.

---

## A METHOD FOR THE DETERMINATION OF ERYTHROCYTE FRAGILITY, USING VAN ALLEN HEMATOCRIT TUBES FOR THE MEASUREMENT OF CHANGES IN VOLUME OF THE CELLS IN HYPOTONIC SALT SOLUTIONS\*

GEORGE M. GUEST, M.D., AND MARY WING, M.S.  
CINCINNATI, OHIO

---

THE procedure here described is designed to facilitate the measurement of swelling of erythrocytes in hypotonic salt solutions, making this measurement practicable in clinical studies of osmotic resistance or fragility of the red blood cells. The method is fairly rapid and requires only a small amount of blood.

*Apparatus.*—Either the original form of Van Allen (1925) hematocrit tube or the modified form recently described (Guest, 1938), providing for automatic volume adjustment of the blood sample, can be used. In most instances from 12 to 16 tubes should be used, but this number can be increased or decreased to suit the needs of individual experiments. For centrifugation of the hematocrit tubes we have used the International centrifuges, Type SB, Nos. 1 and 2. For the original type of Van Allen tubes we have used for the most part the No. 1 centrifuge, with the 8-place head carrying Babcock test metal cups. The short Babcock test cups are fitted with wooden cylinders drilled perpendicularly with

---

\*From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati.

Received for publication, August 2, 1938.

4 holes, each hole large enough to accommodate the stem of the Van Allen tube with its sealing clip. Alternatively, these Van Allen tubes can be carried in the No 1 centrifuge in 10 ml centrifuge tube holders with the 8 place head and 3 place tinnion carriers. For the modified Van Allen tube (longer and somewhat less sturdy) we use the 15 cc centrifuge tube holder, this holder being deep enough to support the hematocrit tube well above the bulb. These are placed in the No 2 centrifuge with the 8 place head and 3 place tinnion carriers, thus, 24 tubes can be centrifuged at once if needed.

*Solutions*—Salt solutions of different concentrations (indicated in Table I) are prepared by delivering from a burette different amounts of a 5 per cent stock solution of sodium chloride C P into 200 cc volumetric flasks. These are diluted to volume with distilled water and transferred to tightly stoppered Pyrex bottles. Such solutions, if kept in a refrigerator will remain unchanged for several months. At the time of use, needed amounts of these solutions are transferred to a series of small vials into which the Van Allen tubes can be dipped for diluting the blood samples.

*Blood Samples*—Either defibrinated or heparinized blood can be used but oxalate, citrate, and fluoride anticoagulants should be avoided because of their effects upon the osmotic equilibrium of the cells. If the blood is drawn by venipuncture, both needle and syringe should be dry. Best results are obtained where the volume of cells in the sample is above 30 per cent. Where the volume of cells in a given blood is low, the sample should be centrifuged just sufficiently to throw down the cells, part of the plasma drawn off to bring the volume of cells well above 30 per cent, and the cells then resuspended in the remaining plasma—a glass bead being added to aid the mixing of the cells and plasma. In some instances it may be desirable to use a suspension of red blood cells, freed of their plasma, in 0.9 per cent sodium chloride solution (see Discussion). To obtain this, the blood sample is centrifuged, the plasma drawn off completely, and the cells are suspended in an approximately equal volume of the salt solution. One half to 1 cc of either whole blood or such a cell suspension will easily suffice.

*Procedure*—The manner of using both the original and the modified Van Allen hematocrit tubes is described more fully elsewhere (Guest 1938). In using the original type of tube, blood is drawn into the stem up to the 100 mark. In using the modified tube, blood is drawn into the measuring stem until the capillary tube is completely filled. With either tube, the tip is then wiped and the blood drawn up slightly into the bulb, the appropriate salt solution is drawn into the bulb until it is nearly filled—leaving a small air bubble to facilitate mixing of the cell suspension—and the spring clip sealing device is fitted over the graduated stem. The dilution with 0.9 per cent sodium chloride in the first tube of the series should be made in duplicate or triplicate, since the cell volume determined in the first tube will be used as a basis for comparison of the values determined in the other tubes. For the subsequent dilutions, only single tubes need be used. The successive steps in the changing concentrations of the salt solutions used in the series, decreasing by 0.1 to 0.025 per cent, can be chosen to fit the needs of individual experiments. The decrements in the concentrations should be small throughout the range where hemolysis occurs, and in the tubes

just before the first trace of hemolysis becomes visible. This, of course, can be anticipated better when making repeat examinations of the blood of an individual subject. The filled tubes are left resting on their sides (a wire test tube rack turned on its side is convenient) for one to two hours at room temperature. They are then centrifuged until the cells are packed to constant volume. In the centrifuges designated above, a speed of 2,500 to 3,000 r.p.m. for fifteen minutes is easily sufficient.

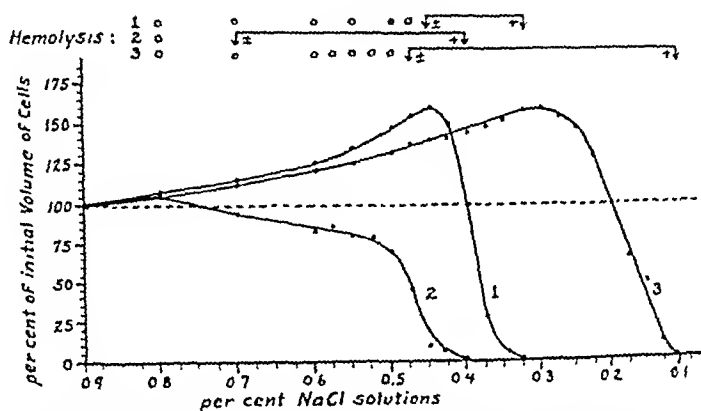


Fig. 1.—Hemolysis and changes in volume of red blood cells in hypotonic salt solutions. Observations on three conditions characterized by different fragilities of the red blood cells: 1, normal (representing the values listed in Table I); 2, familial hemolytic icterus; and 3, erythroblastic anemia (Cooley's anemia).

*Notation of Results.*—After centrifugation, the degree of hemolysis (or its absence) in each tube is read from the color of the supernatant fluid in the bulb, and recorded by suitable symbols from zero, a trace, etc., to complete hemolysis, according to the usual practice in other methods for determining fragility of the red blood cells. The height of the column of packed cells in each tube is read from the graduations on the stem and recorded as shown in Table I. The values for the volume of cells in the several tubes are then divided by that of the first tube and multiplied by 100, in order to convert the readings to percentages of the volume of the cells in 0.9 per cent sodium chloride. For graphic representation, the latter values are plotted as illustrated in Fig. 1, and the degree of hemolysis at each point in the series is indicated by suitable symbols across the top of the chart.

#### DISCUSSION

The values listed in Table I, obtained according to procedure on a sample of normal human blood, illustrate the method of notation herewith described. Fig. 1 illustrates observations which have been made on samples of blood from subjects with conditions characterized by different fragilities of the red blood cells: 1, normal (curve No. 1 represents the example given in Table I); 2, familial hemolytic icterus; 3, erythroblastic anemia (Cooley's anemia). The cells of the patient with hemolytic icterus showed the increased fragility that is characteristic of this condition, whereas the cells of the patient with erythroblastic anemia showed an increased resistance to hemolysis by the hypotonic salt solutions. Further studies of these and other types of bloods will be reported in later communications.

TABLE I

NOTATION OF RESULTS HEPARINIZED NORMAL HUMAN BLOOD DILUTED IN VAN ALLEN  
HEMATOCRIT TUBES WITH SOLUTIONS OF SODIUM CHLORIDE OF DIFFERENT  
CONCENTRATIONS

TUBE NO	NaCl PCT CENT	VOLUME OF CELLS		HEMOLYSIS
		READINGS PCT CENT	PERCENTAGE OF NO 1	
1	0.900	39.6	100	0
2	0.800	42.2	107	0
3	0.700	45.2	114	0
4	0.600	49.4	125	0
5	0.550	52.1	134	0
6	0.500	58.7	148	0
7	0.475	61.2	155	0
8	0.450	63.9	161	±
9	0.425	59.3	150	+
10	0.400	38.8	98	++
11	0.375	11.2	28	+++
12	0.350	2.0	5	+++
13	0.325	0.0	0	Complete

The points on the curves represent the volumes of cells in the several salt solutions in percentage of their initial volume in 0.9 per cent sodium chloride solution. The curves first rise with swelling of all the cells up to the point where the first trace of hemolysis appears, and then fall when the loss of cells by hemolysis exceeds the increases in volume of the unhemolyzed cells. A generally accepted view of hemolysis is that the individual erythrocyte obeys the all or none law, i.e., when a cell is hemolyzed, all the hemoglobin escapes at once. Since the cells in any given sample do not all behave alike, the curves have variably rounded peaks and more or less gradual downward slopes. Thus, the shape of each curve indicates the relative tendencies of the cells in the given blood sample to disintegrate in salt solutions of different concentrations.

While this procedure is intended to serve most purposes of clinical study, details of the method can be changed easily to suit the needs of investigations of special problems concerning erythrocyte fragility. Solutions of other salts and salt mixtures, or of nonelectrolytes, can be used. Instead of the sodium chloride solutions of graded tonicities usually employed in cell fragility tests, Wiseman (1932) has recommended the use of hypotonic solutions prepared by diluting plasma, drawn from the cells to be tested with distilled water. The comparison of the fragility of cells in such hypotonic serum solutions with their fragility in simple salt solutions should be instructive, inasmuch as the blood plasma may contain substances which affect the osmotic resistance of the erythrocytes—either favoring or inhibiting hemolysis. With marked prejudice the color of the supernatant fluid may obscure the readings of the first traces of hemolysis. In such cases it may be advisable to get rid of the plasma in the manner described under preparation of the blood sample, and to work with a suspension of the cells in salt solution. It is to be noted, however, that the behavior of cells washed one or more times with salt solution may differ from that of unwashed cells.

## SUMMARY

A method for determining red blood cell fragility in hypotonic sodium chloride solutions is described, wherein Van Allen hematocrit tubes are employed to measure changes in volume of the erythrocytes in each solution used in the

hemolytic series. The volumes attained by the cells in increasingly hypotonic solutions are expressed in percentage of their initial volume, and recorded graphically. Hemolysis also is read in the same tubes, and the points of beginning and complete hemolysis recorded according to usual practice.

## REFERENCES

- Guest, G. M.: Modified Van Allen Hematocrit Tube Providing for Automatic Volume Adjustment of the Blood Sample, *J. LAB. & CLIN. MED.* 24: 75, 1938.
- Van Allen, C. M.: An Hematocrit Method, *J. LAB. & CLIN. MED.* 10: 1027, 1925.
- Wiseman, B. K., and Bierbaum, O. S.: A New Method for Determining the Fragility of Red Blood Cells, *Proc. Soc. Exper. Biol. & Med.* 29: 835, 1932.
- Beebe, R. T., and Hanley, L. P.: A Method for Measuring the Fragility of Erythrocytes in Salt Solution, *J. LAB. & CLIN. MED.* 21: 833, 1936.
- Castle, W. B., and Daland, G. A.: Susceptibility of Mammalian Erythrocytes to Hemolysis With Hypotonic Solutions: A Function of Differences Between Discoidal Volume and Volume of a Sphere of Equal Surface, *Arch. Int. Med.* 60: 949, 1937.
- Daland, G. A., and Worthley, K.: The Resistance of Red Blood Cells to Hemolysis in Hypotonic Solutions of Sodium Chloride. Observations in Blood Disorders, *J. LAB. & CLIN. MED.* 20: 1122, 1935.
- Harvey, W. F.: Studies in Method and Standardization of Blood Examination. IV. Estimation of Erythrocyte Fragility and a Normal Standard, *Edinburgh M. J.* 44: 100, 1937.
- Lepeschkin, W. W.: Fragility of Red Corpuscles and Its Determination in Clinical Work, *J. LAB. & CLIN. MED.* 17: 1250, 1932.
- Ponder, E.: Mammalian Red Cell and the Properties of Hemolytic Systems, Berlin, 1934, Borntraeger.
- Ponder, E., and Saslow, G.: The Measurement of Red Cell Volume. III. Alterations of Cell Volume in Extremely Hypotonic Solutions, *J. Physiol.* 73: 267, 1931.
- Saslow, G.: On the Supposed Partial Liberation of Haemoglobin From the Mammalian Erythrocyte, *Quart. J. Exper. Physiol.* 19: 329, 1929.
- Warburg, E., and Winge, K.: A Method of Determining Osmotic Pressure of the Cations in the Serum and a Few Preliminary Results Obtained by It, *Acta med. Scandinav. Supp.* No. 26, p. 500, 1928.

## A MORE RAPID METHOD OF GUINEA PIG INOCULATION FOR THE DIAGNOSIS OF TUBERCULOSIS\*

CARRIE I. WOOLSEY, M. A., CHICAGO, ILL.

FORMER methods of guinea pig inoculation for the diagnosis of tuberculosis have the following unsatisfactory factors: the length of time before a report may be expected, the possible death of the guinea pig due to the presence in the exudate of pathogenic bacteria other than tubercle bacilli, the possibility of the occurrence of a tuberculous guinea pig from contaminated surroundings.

Since results obtained by the intra-ventaneous injection of guinea pigs with tuberculous exudates recently reported by Herrold and Woolsey<sup>1</sup> tend to overcome these defects in the old procedures, the intra-ventaneous method has been adopted in the laboratory at Cook County Hospital and was used in the studies included in this report.

### METHOD OF PROCEDURE

After shaving a small area on the side of the abdomen of a guinea pig, 0.4 cc. of the infective material is injected intra-ventaneously. Within seven to twenty-one days, or in unusual cases later, a nodule appears at the site of the injection, which averages about 2 mm. in diameter. The nodule is incised and smears are examined for the presence of acid fast bacilli. Each guinea pig is observed every week and a notation made indicating its condition on each date. If two weeks pass between examinations, a closed nodule may become craterlike and consequently more easily and definitely proved positive. However, the earlier diagnosis would be missed by the delay. It should be noted, the early smear may demand a reasonable period of search to find the organisms. In an occasional case, the third or fourth examination of the pustule may be necessary before the organisms are found. As soon as the inguinal lymph nodes have formed on the side of the injection, the guinea pig may be killed, and an autopsy performed to verify the early report.

One guinea pig may be given more than one injection of the suspected fluid collected at various intervals but can be diagnosed and reported individually. In a similar manner two exudates from different sources of a given patient may be injected on opposite sides of a guinea pig, and each may be diagnosed and reported separately.

The following reports are illustrative of some of the advantages of the intra-ventaneous injection of guinea pigs.

A G—Spinal fluid of puncture December 14 reported positive in fourteen days.

Spinal fluid of puncture December 15 reported positive in twenty-one days.

\*From the Department of Pathology and Bacteriology, Cook County Hospital, Chicago.  
Received for publication August 3, 1938.

Both processes developed on the same pig independently of each other. Regional adenopathy with deep hard nodules in the groin developed in three and five weeks, respectively. At five weeks the guinea pig was killed, and the autopsy revealed findings typical of tuberculosis.

The diagnosis had been reached in fourteen days.

D. S.—March 1, spinal fluid was received and cultured on Herrold egg yolk agar medium.<sup>2, 3</sup>

March 11, colonies of acid-fast bacilli were reported recovered from the culture.

March 11, a second spinal fluid was received and injected into a guinea pig. On the opposite side of the same pig, 0.4 c.c. of a suspension of the Herrold culture growth was injected.

March 22, a nodule produced by the suspension was reported positive for tubercle bacilli.

March 30, the second spinal fluid was reported positive after nineteen days.

Regional adenopathy developed on both sides, and the subsequent autopsy findings at five weeks were typical of tuberculosis.

In this way a culture may be readily verified.

J. W.—On January 20, specimens of spinal fluid and pleural fluid were injected on opposite sides of a guinea pig.

The spinal fluid was reported positive in twenty-three days, but the site of the pleural fluid injection remained negative throughout. Autopsy findings after three months revealed regional adenopathy only on the left side, where the spinal fluid had been injected, and generalized tuberculosis.

R. S.—Pleural fluid was reported positive in six days.

Pericardial fluid was reported positive in twenty days.

Autopsy at four weeks revealed regional adenopathy on both sides and generalized tuberculosis.

Urine from both kidneys of a patient has frequently been injected into opposite sides of a guinea pig to ascertain if one or both organs are involved.

Excepting sputa, secondary invaders in the exudates have not killed any pigs or interfered with the development of tuberculous lesions at the site of injection when given intracutaneously. A small abscess soon develops in such cases from which *Staphylococcus aureus*, hemolyzing streptococci, and *B. coli* among others have been isolated. Occasionally, acid-fast bacilli have been found even in this early abscess, but more often it heals over readily and is followed by the later nodule from which only the acid-fast bacilli are recovered.

Untreated sputa injected into the guinea pigs intracutaneously have been proved to contain tubercle bacilli in two weeks, although the direct examination of the sputum had failed to demonstrate them. Occasionally other bacteria in the sputum will kill the guinea pig, but if *B. fusiformis* is present in notable numbers, the anaerobe will burrow through the deep layers of the skin and eventually the guinea pig will have to be killed before the question of tuberculosis is settled.

Since the initial lesion is localized, a diagnosis may be made before or without an autopsy of the guinea pig. Such a localized initial lesion with a subsequent regional adenopathy is the proof of a positive injected animal.

In the total number of 173 guinea pigs included in this series, tubercle bacilli were recovered 72 times; or in 40 per cent of the exudates studied during

this period. They represent 27 different human exudates that were suspected of containing tubercle bacilli.

The average time for a positive diagnosis was twenty days, ranging from six to thirty one days.

Twenty five per cent of the positives were so reported within fourteen days.

Of the 173 guinea pigs of the series 4 died of unknown cause, 1 on the sixth day, 2 on the eighth, and 1 on the twelfth day respectively, and autopsies revealed nothing suggestive of tuberculosis. One was killed because of infection with *B. fusiformis*.

The first 36 guinea pigs reported negative were killed after six to eight weeks and autopsies were performed. They were in excellent condition and the autopsies revealed nothing suggestive of tuberculosis. Therefore, the subsequent 60 negative guinea pigs of the series were kept as breeding stock since the injection had not retarded them in any way.

#### SUMMARY

The average time for the diagnosis of the tuberculous exudates by means of the intracutaneous injection of guinea pigs is three weeks in comparison with the six to eight weeks required by the old subcutaneous route.

Of the 173 guinea pigs injected in this series only 5 died before the presence or absence of tubercle bacilli in the fluid could be determined.

Since the diagnosis is made from the initial lesion and substantiated by regional adenopathy and autopsy, there is little chance of a spontaneously infected guinea pig not being recognized as such.

Those guinea pigs that remain negative for six weeks may be kept as breeding stock.

#### REFERENCES

- 1 Herrold, R. D., and Woolsey, C. I. Observations on Guinea Pigs Given Intracutaneous Injections of Tuberculous Exudates, Arch. Path. 26: 374, 1938.
- 2 Herrold, R. D. Egg Yolk Agar Medium for the Growth of Tubercle Bacilli, J. Infect. Dis. 48: 236, 1931.
- 3 Woolsey, C. I. The Diagnosis of Tuberculosis. A Comparison of Results Obtained From Herrold Egg Yolk Agar Medium and With Inoculation of Guinea Pigs, J. Infect. Dis. 49: 177, 1931.



## A SIMPLE METHOD FOR THE ASPIRATION OF BONE MARROW\*

MAURICE MORRISON, M.D., AND A. A. SAMWICK, B.S., M.D.  
BROOKLYN, N. Y.

THE study of the hematocytology of the bone marrow has become so necessary in the diagnosis of diseases of the hemopoietic system that a method for the aspiration of the marrow of the sternum must be simple, adequate, and devoid of danger. Furthermore, it must be one which allows repeated studies with the least amount of trauma.

Autopsy formed the only source of material for the study of the bone marrow until Ghedini (1908)<sup>1</sup> trephined and scraped the tibia of patients for diagnosis. Seyfarth (1922)<sup>2</sup> suggested the sternum as the site for biopsy since it was safe and the marrow was active. Modifications of the original technique have been presented,<sup>3</sup> but the customary use of the trephine prevented its universal application.

The examination of the bone marrow by mere aspiration of its contents, which was devised by Pianese as early as 1903,<sup>4</sup> and neglected because it did not supply material for the study of sections, was revived by Arinkin (1929).<sup>5</sup> With the slight modifications suggested by Sonnenfeld,<sup>6</sup> Arjeff,<sup>7</sup> Baserga,<sup>8</sup> and Reich<sup>9</sup> this method has since been widely used in many clinics.

The method that is presented here is as simple to do as lumbar- or venepuncture. It entails no greater psychic or physical trauma to the patient than either of these procedures. It consists of the use of a special steel needle,† 18 gauge, 2 inches in length, the point of which is so beveled that the left side is slightly higher than the right (Fig. 3). It is just as unnecessary to use local anesthesia, as it would be to resort to the latter for venepuncture. In more than 250 aspirations<sup>10</sup> we have had no instance in which the patient has been unduly annoyed by this procedure.

*Procedure.*—1. The instrument tray bearing the following is placed out of sight of the patient: (a) a 5 c.c. syringe (to draw up bone marrow); (b) an aspiration needle; (c) a hemostat; (d) iodine, alcohol, stick sponges, and sterile towels.

2. The region about the angle of Louis (sternomanubrial junction) is prepared with iodine and alcohol; the surrounding region is draped with 2 sterile towels and general asepsis is observed.

3. The point of entrance at the midpoint of the sternum just below the angle of Louis (Fig. 2) is then localized.

4. With the needle between the thumb and index finger in screwdriver fashion, at a point about one-half inch from the end, so that its fulcrum or

\*From the Division of Hematology of the Departments of Medicine and Laboratories of the Jewish Hospital of Brooklyn.

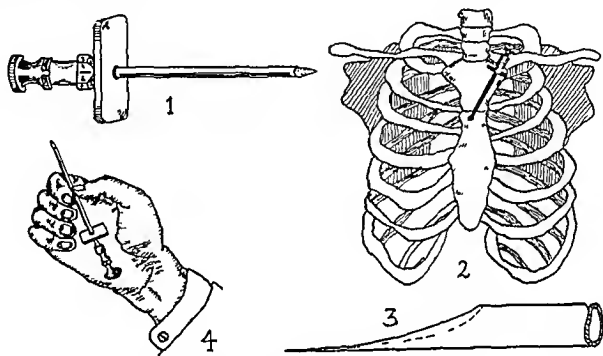
Received for publication, August 12, 1938.

†Needle made by Becton, Dickinson and Co., No. 484 NT. (Fig. 1).

base rests snugly in the palm of the hand (Fig 4), the skin is pierced almost perpendicularly and the periosteum is entered

5 Downward pressure is exerted, turning the needle in screwdriver fashion

6 In an elderly patient a sudden "give" is felt, indicating that the bone marrow cavity has been entered. In younger individuals the "give" is not readily apparent and one must desist from persistent pressure and work slowly and carefully, turning the needle from side to side and aspirating occasionally



Figs 1-4—1 Actual size of needle (diagrammatic) 2 Site of aspiration below angle of Ludwig 3 Sketch illustrating point of needle with bevel of left side higher than the right 4 Needle held firmly in the palm of the hand (screwdriver fashion)

7 The needle is *not* depressed at this stage to avoid breakage

8 The obturator is removed, and the 5 cc syringe is attached. A minimum amount of bone marrow (usually 1 to 3 drops) is withdrawn to avoid excessive dilution with peripheral blood

9 On clean prepared slides thin smears of the marrow are made. At the same time smears of peripheral blood are prepared and all are stained by the usual methods. These may be studied with as much precision as ordinary blood smears

*Advantages of This Method*—1 It can be used in all cases because it minimizes mental and physical trauma

2 It can be oft repeated

3 The needle cannot break under ordinary circumstances but if it should, it would only break at the junction of the base with the needle, and can easily be removed with a hemostat

4 The higher left edge produces a "gouging of the bone" so that entrance into the bone is smooth

5 The screwdriver motion avoids the "straight push" method which is fraught with danger and favors breaking of the needle

6 When difficulty in obtaining post mortem examinations is anticipated, bone marrow smears can be made in the preagonal state

*Disadvantages of Other Methods.*—1. Usually other methods are too elaborate and cumbersome and frighten the patient.

2. Local anesthesia adds further risk with attendant discomfort. Sometimes the patient may have an idiosyncrasy to novocain.

3. More involved procedures do not yield more information,<sup>11</sup> and, in addition written consent is usually required.

4. The "push method" involves great risk whether the needle is controlled by one or two hands.

Aspiration of the bone marrow is useless in the hands of one who is unable to identify the cells and interpret the findings.

#### REFERENCES

1. Ghedini, G.: *Punctura esplorativa del midollo osseo*, Clin. med. ital. 47: 724, 1908.
2. Seyfarth, C.: *Die Sternuntrepanation*, Arch. f. Schiff's - u. Tropen-Hyg. 26: 337, 1922.
3. (a) Morris, L. M., and Falconer, E. H.: *Intravital Bone Marrow Studies*, Arch. Int. Med. 30: 485, 1922.  
(b) Tuohy, E. O., and Gillespie, M. G.: *A Trephine Modified to Secure Bone Marrow (Sternal) Biopsies*, J. A. M. A. 104: 404, 1935.
4. Pianese (1903), Quoted by Caronia, G.: *Pediat.* 30: 607, 1922.
5. Arinkin, M.: *Die Intravital Untersuchungs Methodik des Knochenmarks*, Folia haemat. 38: 233, 1929.
6. Sonnenfeld, A.: *Zur Technik der Sternalpunktion*, Deutsche med. Wchnschr. 54: 1359, 1928.
7. Arjeff, M. J.: *Zur Methodik der Diagnostischen Punktion des Brustbeines*, Folia haemat. 45: 55, 1931.
8. Baserga, A.: *La tecnica della sternopunctura*, Riforma med. 50: 1707, 1934.
9. Reich, O.: *Modified Technic for Sternal Puncture and Its Value in Hematologic Diagnosis*, J. CLIN. & LAB. MED. 20: 286, 1934.
10. Morrison, Maurice, and Samwick, A. A.: *Clinico-Hematological Evaluation of Sternal Aspirations*. Unpublished data.

## A COMPARATIVE STUDY OF CERTAIN METHODS OF MEASURING BLOOD PRESSURE\*

JOSEPH B. Cady, M.D., AND J. F. HERRICK, Ph.D., ROCHESTER, MINN.

THE standard method of measuring the mean arterial blood pressure in the dog is by a direct communication between a cannulated blood vessel and a mercury manometer. The number of times that one can thus measure the blood pressure in the same intact dog is limited to the number of arteries available because the cannulated artery has to be ligated at the end of each experiment. Another important objection to the standard method is that true pulse pressures cannot be obtained. The auscultatory method has been used by several investigators for observing pulse pressures. In our experience, however, this auscultatory method has not been entirely satisfactory. The systolic pressures are always lower and the diastolic pressures definitely higher than those obtained by other methods. If one is interested in differences of pressure only, the auscultatory method may be adequate.

The "hypodermic" manometer developed by Hamilton Brewer and Brotman has proved to be a most satisfactory method for measuring blood pressure. It has been used in this laboratory for three years. To those inexperienced with the method, however, the values appear too high, and it was because of this criticism that these comparative studies of the reliability of the instrument were made preparatory to certain studies of differential pressure, which will be reported later. We have endeavored to check the hypodermic manometer against the mercury manometer (1) by comparison with mean pressures obtained by the standard method, using a hypodermic needle instead of a cannula (the hypodermic needle offers the necessary constriction for damping the oscillations, thus permitting registration of the true mean blood pressure), and (2) by comparison with systolic and diastolic pressures obtained by auscultation.

The main portion of our investigation has been concerned not so much with absolute basal pressures as with the difference in pressure between the carotid and femoral arteries. The present study is, therefore, not intended to present values for the "normal" blood pressure of dogs, but only to check the reliability of the apparatus. We did not take the precautions which others have taken to insure the lowest possible pressures, such as maintaining absolute quiet and a soft bed for the animals, withholding food and administering morphine. For our purposes all that was essential was that the animals be sufficiently trained to lie quietly on the table and submit without struggling to direct arterial puncture. We have found that morphine alone will often

\*From the Mayo Foundation, Rochester, Minn.  
Received for publication August 15, 1935.

reduce the femoral blood pressure from 10 to 15 per cent. In addition we have found that, by more strict adherence to the aforementioned precautions, we can obtain figures with the Hamilton manometer which approach those published by other workers.

The Hamilton optical manometer has been devised to record blood pressure by direct puncture of the arteries of trained, unanesthetized animals. It has been constructed to conform to the requirements for accurate measurement set forth by physiologists and physiologists, namely: the possession of a membrane of sufficient sensitivity and high frequency, in a system which is otherwise practically nonexpansile. In our experience, under the conditions mentioned, this manometer has recorded figures which are consistently and considerably higher than those which have been quoted in the literature. Thus, in the case of one dog whose femoral blood pressure has been recorded fifteen times during the past year, the systolic and diastolic variations have been 170 to 215 and 86 to 114 mm. of mercury, respectively (average 196/99). The average of 83 observations on 24 dogs whose blood pressure was similarly recorded was 201 mm. systolic and 107 mm. diastolic. We would like to re-emphasize the fact, however, that these figures are not to be taken as basal femoral pressures.

#### METHODS

To compare the mean pressures a 20 gauge needle was inserted into one of the femoral arteries of an unanesthetized dog and connected by rubber tubing to the usual type of U-tube mercury manometer. At the same time a second 20 gauge needle, connected by lead tubing with the Hamilton manometer, was inserted into the contralateral femoral artery. A series of several readings of the mean pressure was made and recorded from the mercury manometer at the same time that a series of pulse pressure tracings was photographically recorded from the optical manometer. The mean pressures were calculated from the latter tracings by graphic integration of the pulse pressure curves with a planimeter, since it has been repeatedly demonstrated that the mean pressure is the integrated rather than the arithmetic mean of the systolic and diastolic pressures. In some series of observations, variations in pressure were produced by injections of histamine intravenously.

Further observations were made to compare the direct and indirect methods of recording systolic and diastolic blood pressure. For this purpose a pneumatic cuff was constructed (after the manner described by Freeman and Page) which was 5 by 14 cm. in size and incised in a cloth cuff 35 cm. long. This cuff was wrapped snugly about the animal's leg in such a manner that its lower border covered a small portion of a 2 cm. Bowles type stethoscope chest piece which was attached to the shaven skin over the posterior tibial artery above the ankle by means of an adhesive paste. The systolic and diastolic pressures were read and recorded from a mercury Banmanometer at the same time an assistant photographically recorded the femoral pulse pressure by a Hamilton manometer connected with the femoral artery of the opposite leg. A series of such simultaneous observations (about thirty seconds apart) was made on each animal.

TABLE I

BILATERAL ARTERIAL BLOOD PRESSURES RECORDED SIMULTANEOUSLY WITH THE HAMILTON OPTICAL MANOMETER

ARTERIES	OBSERVATION	SYSTOLIC (MM HG)		DIASTOLIC (MM HG)	
		RIGHT	LEFT	RIGHT	LEFT
Femoral	1	227	226	108	111
	2	231	234	112	115
Femoral	1	204	202	106	103
	2	209	207	108	105
Femoral	1	193	189	103	99
Femoral	1	198	198	122	125
Femoral	1	185	186	88	90
Carotid	1	176	179	125	126
	2	171	175	121	122
	3	173	178	127	129
Brachial	1	140	148	109	108
	2	142	146	115	114

## RESULTS

To demonstrate the sphygmie equality of the corresponding vessels on the two sides of the dog, observations were simultaneously recorded as shown in Table I. From these data we feel safe in assuming that under normal conditions, the two femoral arteries have the same blood pressure. A comparison of the mean pressures obtained by the methods described is recorded in Table II. In our series of observations the greatest difference between any two simultaneously recorded figures was 10 mm. of mercury and the average difference was 2 mm., there was no apparent consistency about which method gave the higher values. Had the mean pressures been determined in the usual manner, that is, by taking half the sum of the systolic and diastolic values, the average for the series would have been 153 mm. of mercury, 30 per cent higher than the figure obtained with the mercury manometer.

TABLE II

A COMPARISON OF OBSERVATIONS OF MEAN BLOOD PRESSURE AS DETERMINED WITH THE HAMILTON OPTICAL MANOMETER AND THE MERCURY MANOMETER

OBSERVATION	MEAN PRESSURE (MM. HG)	
	HAMILTON MANOMETER	MERCURY MANOMETER
1*	125	126
2*	79	74
3*	122	114
4*	120	130
5*	101	96
1	127	122
2	120	125
3	125	127
4	125	125
1	128	125
2	133	123
3	124	121
4	126	117
1	123	117
1	137	134
1	120	114
2	112	116
2	122	120
4	120	117
Mean	120	118

\*Changes in pressure brought about by the intravenous injection of histamine

Table III shows a comparison of the systolic and diastolic pressures recorded optically from the femoral artery and simultaneously observed by auscultation over the contralateral posterior tibial artery. We have so far found it impossible to obtain readings by the auscultatory method that will compare closely with those recorded by the hypodermic manometer. Using the optical instrument on human subjects, Hamilton and his associates found that the systolic values were 3 to 4 mm. higher and the diastolic about 9 mm. lower than the figures obtained simultaneously by the usual clinical method. By the methods which we have used on dogs, we have, in most instances, been unable to find a correlation as close as this. This has been true whether we used the dorsalis pedis artery (as recommended by Freeman and Page) or the posterior tibial artery, and regardless of the width of the cuff or the size of the stethoscope bell. Our closest figures have been secured when larger dogs with relatively larger vessels were used and when the posterior tibial artery above the ankle was employed.

TABLE III

A COMPARISON OF THE INDIRECT (AUSCULTATORY) AND DIRECT (HAMILTON MANOMETER) METHODS OF ESTIMATING ARTERIAL BLOOD PRESSURE

OBSERVATION	SYSTOLIC (MM. HG)		DIASTOLIC (MM. HG)	
	DIRECT	INDIRECT	DIRECT	INDIRECT
1	182	174	100	108
2	187	174	104	110
3	179	178	102	108
1	225	214	115	124
2	222	212	114	120
1	223	220	96	120
2	230	226	102	112
3	220	216	95	116
4	210	202	101	114
5	215	210	102	110
1	206	166	96	92
2	202	164	86	88
3	201	158	90	96
4	205	166	90	88
1	220	194	97	118
2	210	196	93	124
3	206	190	99	120

The generally accepted clinical procedure in ascertaining the diastolic blood pressure is to note the point in the descent of the column of mercury at which the tone of the sounds changes and begins to fade—the fading of the so-called fourth phase. This was found to give values considerably higher than those recorded optically, and it was discovered that a much closer diastolic correlation resulted when the point of total disappearance of the sounds was used (see the next to the last series of observations in Table III).

It is possible that our figures may show a closer correlation than is apparent from the table. The systolic pressure is known to vary at different levels of the vascular tree. Hamilton and his associates found the pressure in the dorsalis pedis artery to exceed that in the femoral artery in man by 25 mm. of mercury. On the other hand, Dawson found that the pressure in the saphenous artery in 2 dogs to be 30 per cent lower than that in the femoral artery. We have recorded the pressure simultaneously in the femoral and

opposite posterior tibial arteries by the Hamilton method in 3 dogs and found the femoral pressure to be definitely higher (10 to 15 mm). From such a small number of observations it is obviously impossible to draw conclusions. However, the data of previous workers and ourselves indicate that one may not be justified in assuming that the systolic pressure in the posterior tibial artery is the same (within the limits of experimental error) as that in the femoral artery.

An observation which we believe to be significant and which was made repeatedly showed that the act of inserting a needle for direct measurements of arterial pressure does not apparently increase the arterial pressure in the trained unanesthetized dog. This observation has been made by taking control auscultatory readings before the needle was inserted into the artery of the opposite leg, with the almost constant finding that subsequent readings are not appreciably elevated above the control readings.

#### SUMMARY AND CONCLUSIONS

A sphygmie equality exists between the two femoral arteries of the dog.

There is a satisfactory correlation between the integrated mean blood pressure, recorded with the Hamilton hypodermic manometer, and the mean pressure, read from a standard mercury manometer.

The values for the systolic and diastolic blood pressures observed over the posterior tibial artery by the auscultatory method did not agree with those for the opposite femoral artery recorded by the hypodermic manometer.

#### REFERENCES

- 1 Hamilton, W. F., Brewer, George, and Brotman, Irving. Pressure Pulse Contours in the Intact Animal. I. Analytical Description of a New High Frequency Hypodermic Manometer With Illustrative Curves of Simultaneous Arterial and Intracardiac Pressures, *Am J Physiol* 107: 427, 1934.
- 2 Freeman, N. E., and Page, I. H. Hypertension Produced by Constriction of the Renal Artery in Sympathectomized Dogs, *Am Heart J* 14: 405, 1937.
- 3 Hamilton, W. F., Woodbury, R. A., and Haiper, H. T., Jr. Physiologic Relationships Between Intrathoracic, Intraspinal and Arterial Pressures, *J A M A* 107: 853, 1936.
- 4 Dawson, P. M. The Lateral Blood "Pressures" at Different Points of the Arterial Tree, *Am J Physiol* 15: 244, 1906.



## ROSE BENGAL EXCRETION AND HIPPURIC ACID SYNTHESIS TESTS OF LIVER FUNCTION: A COMPARISON\*

W. P. STOWE, M.D., SAN FRANCISCO, CALIF.

IN RECENT years numerous tests of liver function have been devised and advocated. In the short series of proved cases here presented several of these tests have been tried. In none of them did the galactose tolerance test prove positive, nor were icteric index and bilirubin findings parallel to the severity of the liver damage as shown at operation or autopsy. Serum Takata-Ara findings became positive at serum albumin-globulin ratios of 2:1 or less, as did also increased average diameters of the red blood cells above 6.9 micra, by the halometer method applied to dried smears.

Our present interest is, however, centered around two of the simplest and apparently most accurate of the liver function tests.

The rose bengal excretion test is that reported by Delprat and Stowe.<sup>1,2</sup> Details may be obtained from articles quoted, or from the eighth edition of Todd and Sanford.<sup>3</sup> Photometric methods of reading the test are also available in publications of Giordano and Eager<sup>4</sup> and of Stowe and Delprat.<sup>5</sup> Both techniques are completely described in the recent revision of *Approved Laboratory Technique* by Kolmer.<sup>6</sup> Briefly, the test is carried out by injecting intravenously 10 c.c. of 1 per cent solution of rose bengal in saline; waiting two minutes for blood stream mixing, and removing and oxalating a sample of blood. Six minutes later a second blood sample is removed and oxalated. Colorimeter comparison of plasma from the two samples shows a 50 per cent decrease of rose bengal in the second as compared to the first, providing the liver is normal. Greater degrees of retention in the blood stream are proportional to decreased liver function.

The hippuric acid synthesis test of Quick<sup>7</sup> is carried out by feeding the patient 6 gm. of sodium benzoate with a light breakfast. The entire next four hours' urine is saved, acidified slightly with acetic acid, evaporated to 100 c.c. if necessary, and, when cool, further acidified to Congo red with full strength hydrochloric acid. After this it is stirred vigorously until precipitation of hippuric acid occurs, and allowed to stand several hours; the crystals are then filtered off through a Büchner funnel, dried, and weighed. To the weight of the crystals thus obtained is added 0.33 gm. per 100 c.c. of filtrate, for hippuric acid still dissolved in this filtrate. The total figure is then multiplied by 0.68 to convert to benzoic acid in terms of which it is reported. A four-hour excretion of 2.5 gm. to 3.5 gm. as benzoic acid indicates normal

\*From the St. Luke's Hospital, San Francisco.  
Received for publication, August 22, 1938.

liver synthesis. Reduction below 2.5 gm, in the presence of relatively normal kidney function, is evidence of liver damage.

Seven cases are presented in Table I. All diagnoses except that for patient 1, were proved at operation or autopsy. The early slight biliary cirrhosis in patient 7 was shown by biopsy at operation subsequent to the last rose bengal reported. The diagnosis in patient 1 was based on history, positive Wassermann findings, prompt response to treatment, and a later follow up of apparently perfect health after a year's treatment. In the table the rose bengal function is reported in percentage of normal liver function, in column two, hippuric acid excreted in four hours as grams of benzoic. In column three, for purposes of comparison with the rose bengal hippuric acid excretion is figured as percentage of normal using 2.5 gm as normal output. In all except patient 7 the similarity of findings of these two simple tests is obvious. In this last patient, in view of the quite rapid return of rose bengal to normal under glucose therapy, the hippuric acid test proved the more accurate from the standpoint of permanent liver cell damage.

TABLE I

PATIENT	PROVED DIAGNOSIS	ROSE BENZAL FUNCTION	HIPPURIC ACID EXCRETION	HIPPURIC ACID FUNCTION
		<i>Per cent</i>	<i>Gm</i>	<i>Per cent</i>
1 Mrs. A	Syphilitic cirrhosis	34	0.9	35
	After 2 mo. treatment	77	1.8	72
2 Mr. U	Carcinoma of head of pancreas	90	2.8	110
3 Mr. V	Subacute hepatitis	50	0.70	30
	Nitrobenzol poisoning			
4 Mrs. P	"Cardiac cirrhosis," rheumatic heart, 14 yr. decompensated	50	1.1	44
5 Mrs. D	Advanced cirrhosis	20	0.3	12
6 Mrs. I	Advanced cirrhosis	20	0.43	17
7 Mrs. C	Stone in common duct, slight biliary cirrhosis	90	2.00	82
	After one month intravenous glucose		Not repeated	---

## SUMMARY AND COMMENT

Liver function values, as determined by two simple tests, are compared in 7 patients with proved diagnoses. One (rose bengal) is a purely secretory test, the other (Quick's hippuric acid synthesis) is a metabolic test. A striking parallelism of these tests to each other and to the liver condition is shown. In view of the close agreement between the two tests it is suggested that the rose bengal be used where speed is of importance (one hour to complete, as compared to twelve hours or more for hippuric acid test). Also it should be used where kidney function is known to be impaired, since this impairment vitiates the results in Quick's test. Where severe jaundice is present and in laboratories where apparatus and reagents are limited the test of Quick is technically the more feasible and the one to be recommended.

## REFERENCES

- 1 Delprat, G. D., and Stowe, W. P. The Rose Bengal Test for Liver Function, J. I. A. B. & Clin. Med. 16: 92, 1931.

2. Stowe, W. P., Delprat, G. D., and Weeks, Alanson: The Rose Bengal Test of Liver Function, *Am. J. Clin. Path.* 3: 55, 1933.
3. Todd and Sanford: *Clinical Diagnosis by Laboratory Methods*, ed. 8, Philadelphia, 1935, W. B. Saunders & Co., p. 391.
4. Giordano, A. S., and Eager, Dean: The Rose Bengal Liver Function Test as Adapted to the Sheard-Sanford Photometer, *Am. J. Clin. Path.* 5: 417, 1935.
5. Stowe, W. P., and Delprat, G. D.: The Rose Bengal Test of Liver Function: Photometric Method, *J. LAB. & CLIN. MED.* 20: 1297, 1935.
6. Kolmer, J. A., and Boerner, F.: *Approved Laboratory Technic*, ed. 2, New York, 1935, D. Appleton-Century Co., Inc., p. 237.
7. Quick, A. J.: The Synthesis of Hippuric Acid: A New Test of Liver Function, *Am. J. M. Sc.* 185: 630, 1933.

## A SIMPLE METHOD OF OBTAINING SPUTUM FOR PNEUMOCOCCUS TYPING\*

JACK ROM, M.D., DETROIT, MICH.

**I**N INFANTS and young children and in acutely ill pneumonia patients who do not expectorate, it is frequently difficult or impossible to obtain sputum for pneumococcus typing. The method of culturing direct pharyngeal and laryngeal swabs is frequently unsatisfactory, while swabs by laryngoscopy and lung suction by means of transthoracic pulmonary puncture are methods not applicable to general usage. For this reason we have employed a method of obtaining material for pneumococcus typing, which we believe is simple, accurate, and practical for use both in the hospital and in the home. It includes the following: 20 to 50 c.c. Luer syringe (50 c.c. preferable); soft rubber catheter (size dependent upon age of patient); small sputum bottle containing 4 to 5 c.c. of sterile saline; and small amount of sterile glycerin.

The catheter is connected to the syringe, a small amount of glycerin is placed on its tip, and the catheter is then passed through the nares back to the nasopharynx and thence downward to the oropharynx. It is then passed slowly down to the larynx. When the catheter reaches the larynx, the patient frequently coughs. The material in the vicinity of the larynx and lower pharynx is then aspirated into the syringe by means of repeated suction. If insufficient material is obtained, the catheter is withdrawn slightly and the aspiration is repeated. After sufficient material has been secured, the catheter is withdrawn, the sterile saline is drawn up into the syringe, and the material flushed out into the sputum bottle.

This method was employed in 72 cases of pneumonia in which sputum was not expectorated. It was possible by this method to obtain sufficient material for bacteriologic study in all of the cases. The material obtained

\*From the Department of Medicine of Wayne University and the Detroit Receiving Hospital.

was examined both by the direct Neufeld method and by mouse injection. The results in these cases are shown in Table I.

TABLE I

CASES	PNEUMOCOCCUS FOUND AND TYPED		NO PNEUMOCOCCUS FOUND		TOTAL
	DIRECT	MURSE	NO OTHER ORGANISMS	OTHER ORGANISMS	
Adults	16	25	1	3	29
Children	20	28	4	1	43
Total	36	63	5	4	72
	6				

## SUMMARY

A method of aspirating sputum for pneumococcus typing is described. The results obtained in 72 cases of pneumonitis indicate that this method is applicable in those cases in which sputum is not expectorated.

## A SIMPLE DEVICE FOR CHANGING THE LIGHT INTENSITY IN THE X RAY ILLUMINATOR

EDWARD J. VAN LIERE, PH.D., M.D., and DAVID W. NORTHUP, PH.D.,  
MORGANTOWN, W. VA.

TWO difficulties are encountered in using an ordinary x-ray illuminator or view box in tracing teleoroentgenograms. First the heat generated by the electric light bulb is apt to injure the film, often too the film becomes so warm that the hand which is used for tracing cannot be allowed to rest on the film. Second, it is not easy, if not impossible to regulate the intensity of light transmitted through the film.

Both of these difficulties can be obviated by the use of a simple and inexpensive device. A foot rheostat, such as is used in operating an electric sewing machine and as shown in Fig. 1, can be inserted in series with the electric light bulb in the x-ray illuminator. Simply by pressing down the foot a wide range of light intensity is instantly available without the necessity of using the hands, so that the latter may be used for tracing the x-ray silhouette. Further, if the film becomes too warm the amount of light can be cut down so that the heat will not injure the film or become too hot for the hand. The electric light goes out automatically when the foot is released, so that the light cannot be accidentally left on and the film ruined.

In making the suggestion that a rheostat be used to vary the intensity of the light transmitted, the authors appreciate that most of the modern view boxes are equipped with mercury vapor lamps. It is obvious that the rheostat

\*From the Department of Physiology, West Virginia University, Morgantown.  
Received for publication August 4, 1938.

could not be used in this type of view box; there are, however, still many view boxes in use which are not so equipped. A criticism which might be raised is that dimming a filament bulb may add an element of red to the transmitted light; many roentgenologists object to this. It is felt that this latter criticism is probably of no great importance if the rheostat is used while tracing cardiac silhouettes.

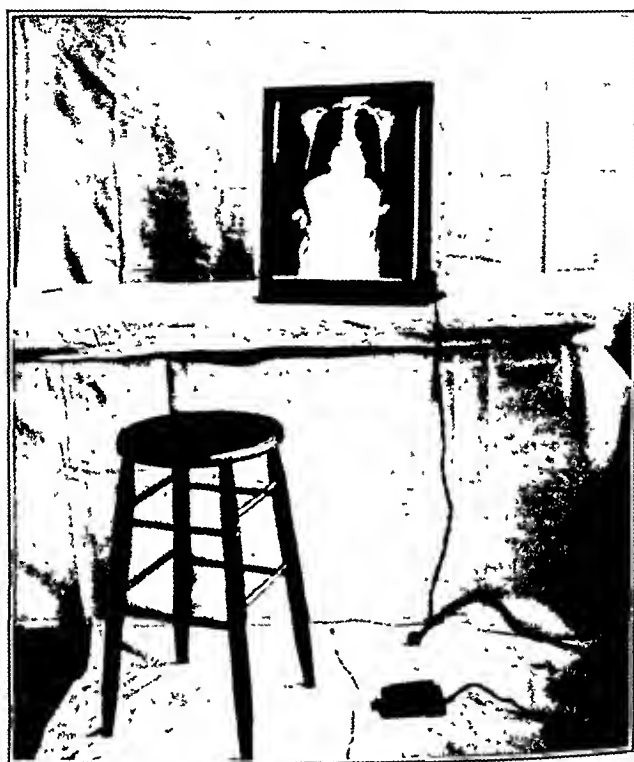


FIG. 1.

The authors feel then that such a device as described is especially useful in tracing the outline of the heart. The change in the intensity of the light often is of distinct help in tracing the apex, and the ability to be able to control the amount of heat generated makes it possible to work leisurely and carefully. Not only, however, is the device useful in tracing teleoroentgenograms, but it is also of use in studying the ordinary x-ray film where it is desirable to change the light intensity.

# A MANUAL OF NEUROHISTOLOGIC TECHNIQUE

OSCAR A. TURNER, M.D. NEW HAVEN, CONN.

(Continued from the April issue page 745)

## CHAPTER V

### STAINING OF FAT

Some form of lipoid stain is generally required in any routine study of the nervous system. These stains are easy to do and give satisfactory results, although there is a tendency for all of them to fade in time. Of necessity all are done on frozen sections and all are mounted in a medium containing no fat solvent.

*Sudan III*—This is a commonly employed stain which is applicable to all types of tissue.

- 1 Frozen sections at 20 microns of formalin fixed tissue are washed thoroughly in distilled water. Tissue embedded in gelatin may be used.

- 2 Transfer sections to 70 per cent alcohol and allow to remain for one minute.

- 3 Stain in a saturated solution of sudan III in 70 per cent alcohol for about twenty minutes.

- 4 Rinse in 70 per cent alcohol.

- 5 Counterstain in hematoxylin.

- 6 Wash well in two changes of distilled water.

- 7 Draw sections on a clean glass slide and mount in gelatin, Arabic sugar or glycerin jelly.

The droplets of fat are colored orange or orange red and stand out fairly well from the light orange background. The cell nuclei are stained, the depth of staining depending upon the type of hematoxylin used and the time of staining in it. Weigert's hematoxylin is a satisfactory stain for the nuclei and Delafield's may be used also. Mayer's hematoxylin gives an excellent nuclear stain. However, if the tissue has been embedded in gelatin before sectioning, it is necessary to use Ehrlich's hematoxylin for the nuclear stain. The preparations are best stored in the dark.

*Heizheimer's Fat Stain* (Sudan IV, Schmalach R, Scarlet Red)—This method, utilizing a derivative of sudan III as a dye, has become widely used and gives results which are superior to the older sudan III method.

- 1 Frozen sections of formalin fixed tissue are cut at about 20 microns.

2. The sections are placed in distilled water.

3. Transfer to the fat stain made as follows:

Place an excess (1 to 2 gm.) of scarlet red dye in a wide-mouthed bottle. To this add the following mixture:

Absolute alcohol	70 c.c.
10% sodium hydroxide	20 c.c.
Distilled water	10 c.c.

Heat and place in the incubator at 37° C. for one hour. Cool in running water, and filter through three thicknesses of coarse filter paper into a large watch glass crystal. Allow solution to stand for twelve hours before using. It should not be more than five to six days old when used.

4. Cover watch glass with a glass plate and heat until the glass plate is sealed with the steam which arises. Allow to stand twenty minutes.

5. Wash in two changes of distilled water, allowing the sections to remain in the last wash for about three hours.

6. Shake in Mayer's hematoxylin diluted with water to about 50 per cent of its original strength. Allow to remain overnight, if necessary.

7. Wash in distilled water and then in tap water for two minutes.

8. Draw sections on clean glass slides under water, dry with filter paper and mount in glycerin jelly.

The fat is seen as small and large globules which are stained a scarlet red. The nuclei stain blue and are more distinct than in the sudan III method. The method has the disadvantage of taking more time to complete, but the results are correspondingly better. However, Hassin recommends placing the sections in about 20 c.c. of water to which 5 or 10 drops of Ehrlich's hematoxylin have been added, and allowing them to remain only fifteen minutes. The Ehrlich's hematoxylin is necessary if the tissue has been embedded in gelatin before sectioning. Otherwise, any hematoxylin may be used. The criticism has sometimes been raised that heating the sections tends to disperse or displace the fat globules. This will not take place if the heating is not carried to an excess. Filtering the staining solution before use helps prevent the formation of precipitates. The solution should always be kept covered to prevent evaporation.

*Nile Blue Sulfate Stain.*—Occasionally one is interested in obtaining an insight into the nature of the lipoidal substances contained in the cells which take the fat stain. Although it is obvious that no one single method is specific for any one type of lipid and that a single fat globule may contain several different lipid substances, the method of Lorrain Smith has proved of value in histochemical investigations. The method is as follows:

1. Frozen sections of formalin-fixed tissue are washed thoroughly in distilled water.

2. Sections are placed for ten minutes in a saturated aqueous solution of Nile blue sulfate.

- 3 Wash for two to three minutes in distilled water
- 4 Mount in glycerin jelly

Fatty acids are stained blue while neutral fats take a pink to red color. Mixtures of these two substances are purple or some shade of purple, depending upon whether the neutral fats or fatty acids predominate. Other lipid bodies such as myelin and lipochrome pigments assume a blue color. Nuclei are stained dark blue and protoplasm a light blue. The stain is not permanent and fades in two to three days. Differentiation may be carried out in 1 per cent acetic acid if the staining is too deep.

## CHAPTER VI

### NONMETALLIC GLIAL STAINS

With the recent additions to our knowledge of the interstitial cells of the nervous system, many new selective staining methods have been devised to demonstrate these cells. The division here into metallic and nonmetallic methods has been made solely for convenience. Certain methods lend themselves more readily to tumor tissue and others more to the study of the degenerative and inflammatory diseases. The special applications of each will be noted in the descriptions of the individual stains.

*Mallory's Phosphotungstic Acid-Hematoxylin Stain*—The best results with this method are obtained on paraffin sections of Zenker fixed tissue. However, good results may also be had from the use of formalin fixed material which has been treated with Zenker's fluid or passed through the Weigert mordants as described by Kennelhan. The stain is one of the simplest and most useful of the nonmetallic methods for the study of glial tissue in tumors.

- 1 Paraffin sections of Zenker fixed tissue are brought to water and treated with Gram's iodine and hypo as usual to remove the mercuric chloride crystals.

- 2 After washing thoroughly, transfer sections to 0.25 per cent potassium permanganate solution and allow to remain for from twenty to thirty minutes.

- 3 Wash well in water and treat in 5 per cent oxalic acid solution until the sections are bleached white. They should remain here at least ten minutes.

- 4 Wash thoroughly in several changes of distilled water.

- 5 Stain in Mallory's phosphotungstic acid hematoxylin solution for twenty four to forty eight hours. The stain is prepared as follows:

Hematein ammonium	0.1 gm
Phosphotungstic acid crystals	20 gm
Water	1000 cc

Dissolve the hematein ammonium in a little water with the aid of heat and when cool add the remaining ingredients. Ripen by the addition of 0.1 cc of hydrogen peroxide or allow to ripen spontaneously for several months.



6. Rinse in water.

7. Dehydrate in 95 per cent alcohol, absolute alcohol, and clear in xylol. Mount in balsam.

Fibrin, fibroglia, neuroglia, and myoglia fibrils are stained blue while the intercellular connective tissue fibrils are stained a pale reddish brown or remain colorless. Collagen tends to take a reddish-pink color in most preparations. The coarse elastic fibers sometimes assume a pale purplish-blue color. If after step 5 the sections are placed in a 10 to 20 per cent alcoholic solution of ferric chloride for one to three minutes followed by thorough washing in water, the intercellular connective tissue fibers are completely decolorized, and the blue-stained glial fibers assume a somewhat deeper hue. The cell nuclei are blue, and the cytoplasm has a pale pink or brownish-pink color. Blepharoplasts may occasionally be stained a pale blue color.

It is essential that the stain be well ripened. The best results are obtained when hematein ammonium is used and allowed to ripen spontaneously for six months to a year. Stain which has been allowed to stand for a year or more gives an intense color to the tissues. Hematoxylin may be used instead of hematein ammonium, but requires twice the amount of hydrogen peroxide to cause ripening.

Formalin-fixed tissue may be employed. For this, Kernohan recommends that the unembedded blocks of tissue be treated four days in Weigert's primary mordant, followed by two days in Weigert's secondary mordant, after which the tissue is embedded in paraffin, sectioned and stained as usual. The same method may be employed on tissue already sectioned and mounted on glass slides. The deparaffinized sections remain in the primary mordant ten to twelve hours, and in the secondary mordant five to six hours. Care should be taken that the sections do not float off the glass slides. Fairly good results may be obtained also if small blocks of the tissue, which has been fixed in formalin or deparaffinized sections, are allowed to remain for three days in Zenker's fixative or 5 per cent potassium bichromate. If Zenker's fixative is used, the sections must be passed through iodine solution and 5 per cent hypo to remove the mercuric chloride crystals. Origanum and other oils cause the stain to fade. When mounted in balsam and not exposed unduly to direct sunlight, the preparations are reasonably permanent.

This method finds its greatest use in the study of tumors, especially the gliomas. It should be noted that the method is not selective for neuroglia, but also stains fibroglia and myoglia as well. The degree of impregnation of the elastic fibers is usually not sufficient to cause difficulty in interpretation.

*Bailey's Ethyl Violet-Orange G Stain for Fibrillary Neuroglia.*—This method was originally intended for use on Zenker-fixed tissue, but Davidoff has described a preliminary treatment whereby good preparations can be obtained on formalin-fixed material. Sections of paraffin-embedded tissue are placed in 50 c.c. of water to which 10 drops of ammonia have been added and allowed to remain for twenty-four hours at room temperature. The ammonia is then washed out with a slow stream of running water for one to two hours, and the sections are treated in Zenker's fluid for twelve to twenty-four hours.

They are then passed through Gram's iodine and hypo to remove the crystals of mercuric chloride and the stain carried out as with tissue fixed originally in Zenker's solution. The method is as follows:

1 Place for three days in a 3 per cent solution of potassium bichromate

2 Rinse in water and transfer sections into a solution of ethyl violet orange G and allow to remain for twelve hours. The staining solution is prepared as follows:

Ethyl violet	10 gm
Orange G	0.5 gm

The dyes should be weighed accurately. Add 100 cc of distilled water and stir thoroughly. Place in the incubator at 37° C. for twelve to twenty-four hours for precipitation to occur. Decant the supernatant fluid, and wash the precipitate several times with distilled water. Place in the incubator to dry. Make a saturated solution of the dried precipitate in absolute alcohol. Use this as a stock solution. For staining use 1 part of the stock solution to 3 parts of 20% alcohol.

3 Blot the sections and agitate quickly in anhydrous acetone

4 Place in toluol for two to three seconds

5 Flood the slide with pure oil of cloves

6 Differentiate in a mixture of 3 parts of oil of cloves and 1 part of 96 per cent alcohol

7 Rinse in pure oil of cloves

8 Pass sections through several changes of toluol and clear in several changes of xylol. Mount in balsam.

The neuroglia fibers appear dark violet against an orange background. The nuclei, fibrin, and erythrocytes are also stained. There is sharp staining of the myelohia and fibroglia, so that the method cannot be depended upon outside of the central nervous system.

In differentiation, after most of the toluol is wiped off the slide the section is flooded with the pure oil of cloves. The slide is then agitated in the differentiating solution and the degree of decolorization is controlled under the microscope. This should be carried to the point where the blood vessels stand out as orange rings. If the decolorization is too rapid, more of the oil of cloves may be added to the differentiating solution. The affinity of the tissues for the dye varies somewhat, the protoplasmic neuroglia of the cortex retaining the stain strongly.

If the tissue has been mordanted for over a week previous to staining there is danger that the myelin sheaths will take the stain. Occasionally this may occur in old formalin fixed tissue.

It is important that the staining solution be prepared carefully. The stock solution, if tightly stoppered, will keep indefinitely. This method has its greatest use in tumor work, and although experience is necessary to obtain the best results the method is reliable and the preparations are excellent for

photography. The stain is not permanent and tends to fade. It should not be exposed to direct sunlight or heat.

*Holzer Glia-Fiber Stain.*—With this method, frozen paraffin, or celloidin sections of formalin-fixed tissue may be used. Alcohol-fixed tissue may be used, but is not recommended. The method is particularly adapted to the study of general neuropathologic material, although useful preparations of tumor tissue can be obtained. The method is one of the best for glia fibers.

(a) *Frozen Sections.*

1. Place sections cut at 15 microns in 50 per cent alcohol and allow to remain for one to two hours.

2. Draw on slide covered with egg albumen-glycerin. Blot dry with filter paper.

3. Wash three times with phosphomolybdic acid solution:

0.5% phosphomolybdic acid, aqueous	1 part
96% alcohol	3 parts

The individual components keep well, but the mixture should be prepared fresh.

4. Blot three times with filter paper soaked in the following alcohol-chloroform mixture:

Absolute alcohol	2 c.c.
Chloroform	8 c.c.
Prepare fresh.	

Firm pressure will prevent the sections from coming off the slides.

5. Cover sections with this same alcohol-chloroform mixture until the sections clear. Do not allow drying to take place.

6. Add crystal violet solution directly to the above.

Crystal violet	1 gm.
Absolute alcohol	2 c.c.
Chloroform	8 c.c.
Filter. Solution keeps well.	

7. Add quickly a 10 per cent solution (aqueous) of potassium bromide by drops until the green sheen which forms disappears. Blot dry with filter paper.

8. Differentiate in the following solution:

Aniline oil	4 c.c.
Chloroform	6 c.c.
1% aqueous acetic acid	1 drop
Filter. Prepare fresh before use.	

It is necessary to control the differentiation under the microscope. When the desired decolorization is attained, flood the slide with xylol and mount in balsam.

It is necessary to work quite rapidly throughout the entire procedure. Because of this, it is convenient to keep the various reagents in small dropper bottles, pouring the solutions over the slides. Coplin jars cannot be used

because of the rapidity with which most of the solutions evaporate. When examining the sections under the microscope, wash out the differentiating solution with xylol lest the decolorization be carried too far. It may be necessary to repeat the process several times before the correct degree of decolorization is obtained.

### (b) Paraffin Sections

1 Place deparaffinized sections for three minutes in the following freshly prepared mixture

0.5% phosphomolybdic acid, aqueous	10 cc
96% alcohol	20 cc

2 Drain off the fluid and cover section with

Absolute alcohol	2 cc
Chloroform	8 cc
Prepare fresh	

3 While still wet, cover with the following staining mixture and allow to remain so for thirty seconds

Crystal violet	0.5 gm
Absolute alcohol	20 cc
Chloroform	80 cc
Filter. This solution keeps well	

4 Replace dye with 10 per cent aqueous potassium bromide solution and wash in this same solution for one minute

5 Differentiate for thirty seconds in

Aniline oil	6 cc
Chloroform	9 cc
Ammonia, 25%	1 drop
Prepare fresh	

6 Wash in xylol. If the section is overstained, repeat differentiation, controlling under the microscope. Mount in balsam after washing thoroughly in xylol.

### (c) Celloidin Sections

Before staining, celloidin sections should be allowed to remain overnight in methyl alcohol. Draw on to glass slides directly from this and blot dry. Place sections for a short time in 50 per cent alcohol, dry and proceed as in the method outlined for frozen sections. Celloidin sections may also be mounted on glass slides before treatment with methyl alcohol. A mixture of equal parts of absolute alcohol and ether may be used also to remove the celloidin.

With the frozen sections and the paraffin embedded tissue the background should be practically colorless. Where celloidin sections have been used the background is usually colored a light blue, but often not uniformly so. Glial cells stain clearly. The fibers and nuclei are colored a deep shade of blue,

the depth of the staining depending upon the degree of decolorization of the tissue. In celloidin sections there may be a tendency for the perivascular connective tissue to take the dye slightly deeper than the background but never enough to interfere with the recognition of the glial fibers. Myelin sheaths, reticulum, axis cylinders, and ganglion cells are not stained. The best results are obtained on frozen and paraffin sections, and these should be used in preference to celloidin sections whenever possible.

Once started, the procedure must be carried through to completion. Precipitates can be avoided by working rapidly and using fresh solutions which are carefully filtered. With the exception of the dye and the potassium bromide solution, all solutions should be prepared fresh.

Warkany's modification, in which frozen sections are used, consists of placing the sections for one to two minutes in equal parts of 1 per cent phosphomolybdic acid and 95 per cent alcohol, washing in a mixture of 4 parts of chloroform and 1 part of 95 per cent alcohol and staining for a few seconds in a mixture of 8 c.c. chloroform, 0.5 gm. crystal violet and 2 c.c. 95 per cent alcohol. The sections are then washed in 95 per cent alcohol for a short time and differentiated in two changes of aniline oil. This method has the advantage of being simpler and less time-consuming than the original Holzer method, but gives results which are less consistent.

*Alzheimer-Mann Stain for Neuroglia.*—This stain, although primarily intended for the study of neuroglia, is important in that it allows the study of both myelin sheaths and axis cylinders in the same preparation. The tissue should be fixed in Weigert's glial (secondary) mordant for at least two weeks. Prolonged fixation has no ill effects.

1. Wash the blocks of tissue for twelve to twenty-four hours to remove the excess copper. Shorter periods of washing, while not deleterious to the stain, will not completely remove the excess copper which may injure the edge of the microtome knife.

2. Cut frozen sections at 10 to 15 microns.

3. Mordant in a saturated aqueous solution of phosphomolybdic acid. The sections may remain in this solution for as long as twelve hours, although one hour is usually enough.

4. Wash quickly in two changes of distilled water.

5. Place in Mann's solution for one to two hours:

1% aqueous methyl blue	35 c.c.
1% aqueous eosin	35 c.c.
Distilled water	100 c.c.

6. Wash rapidly in distilled water.

7. Differentiate in 96 per cent alcohol. This takes place quite rapidly. The white matter should be bright red or pink, and the gray matter a light blue. The differentiation can be controlled by the color of the gray matter.

8. Rapidly dehydrate in absolute alcohol, clear in xylol, and mount in balsam.

Normal myelin sheaths are colorless or pink, depending upon the degree of decolorization. Degenerating myelin sheaths tend to assume a deeper shade and may stain bright red. Normal axis cylinders stain a deep blue, while degenerating axis cylinders are a bright red. The cytoplasm of the glial cells is a pale blue color, while neuroglia fibers take a dark blue stain. Amoeboid glia vary in color from a pale to deep blue and often show stained inclusion bodies. Connective tissue stains a dark blue. In the cortex the differentiation is less clear, the nerve cells take a dark blue color and show relatively little detail.

The method is of particular value in studying spinal cord and peripheral nerves, especially for the pathologic forms of glia and the axis cylinders. Mallory states that the change from blue to red staining of the latter takes place as early as forty eight hours after a lesion while degeneration as demonstrated by the secondary changes in the myelin sheaths (Marchi method) begin to appear only after about seven days.

Although the best results are obtained upon tissue which has been placed directly into the glia mordant, formalin fixed tissue which has been placed in the glia mordant and allowed to remain two weeks may also be used. After the frozen sections are cut, they may be stained at once or allowed to remain overnight in the glia mordant. Instead of utilizing a saturated solution of phosphomolybdic acid, Wertham allows the sections to remain for a half hour in a 0.5 per cent solution, after which they are transferred to a 10 per cent solution for three to four hours. The sections should be handled with glass hooks and care should be taken that they lie flat in the various solutions. Beautiful preparations may be obtained by using this method as a counterstain for the Bielschowsky silver method.

## CHAPTER VII

### METALLIC GLIA STAINS

Although many of the nonmetallic glia stains give excellent pictures of the glial fibers, few give as clear cut and detailed staining of the cell bodies and their processes as do the metallic impregnation methods. Although practically all of the original methods require special fixatives, many modifications make possible the use of formalin fixed tissue. Only those have been selected which give relatively consistent results.

The technique of impregnating the interstitial cells of the central nervous system requires certain care and attention to details which is not usually called for in the staining of other tissues. Although small variations in timing, temperature, and such will often be found necessary to attain the best results by the individual worker, the techniques are more or less standard, and certain factors must be followed closely. The glial cells are particularly susceptible to post mortem change, and as such the tissue should be fixed as soon after death as possible. All glassware must be chemically clean and washed in distilled water prior to use. Unless otherwise indicated, distilled water is to be used for all solutions. Sections should be handled with small glass rods. At the best, metallic stains are apt to be rather capricious, requiring patience and practice.

*Cajal's Gold Sublimate Method.*—This method originally called for tissue fixed in formalin-ammonium bromide. Many preliminary treatments for formalin-fixed tissue have been described, so that at the present time excellent results can be obtained on routine laboratory material. Most of these preliminary treatments follow the work of Globus and depend upon the introduction of bromine in some form into the tissue. However, good results can be obtained occasionally by the use of formalin-fixed tissue without previous treatment, particularly if the tissue has not been fixed in the formalin over two days. The method is one of the simplest and one of the most useful for tumor tissue. Frozen sections, 20 to 30 microns, are used. The method, without previous treatment of the tissues, is as follows:

1. Wash sections in distilled water.

2. Stain from four to eighteen hours (overnight usually) in the following mixture:

1% gold chloride (acid brown)	6 c.c.
1% mercuric chloride	25 c.c.
Distilled water	5 c.c.
Prepare fresh before use.	

This is enough for 6 to 8 average-sized sections. If the sections are particularly large, only 4 should be stained in this amount of solution.

3. Wash in distilled water.

4. Fix for five to ten minutes in 5 per cent sodium thiosulfate. The sections become quite flexible in this solution.

5. Wash well in distilled water.

6. Transfer to 50 per cent alcohol. Mount on glass slides, blot carefully but firmly, complete dehydration in two changes of absolute alcohol, clear in xylol, and mount in balsam. When completely dehydrated the sections are clear when seen with transmitted light, but appear almost opaque with reflected light. They are purplish red or reddish brown in color. They do not fade.

The microscopic picture depends considerably upon the depth of staining. When the staining has been intense, the glial cells, particularly the astrocytes, appear deep purple to black, and the background is a lavender or light reddish-purple color. The glial fibers are of the same purplish-black color. With less intense staining the background is a light lavender, and the individual fibers and cell processes are more distinctly seen. In some instances a certain amount of nuclear detail is evident. Ganglion cells may be faintly impregnated and the capillaries may be outlined by the perivascular extensions of the astrocytes which take the stain deeply.

The length of time required for complete staining varies considerably. When the sections have assumed a purple color, it is best to remove one and examine it under the microscope. In the wet preparation, with the low-power lens of the microscope, the astrocytes should stand out as deeply stained black stars. The affinity for this stain varies with different tissues, so that the

sections should be watched to prevent overstaining. It is not necessary to keep the staining solution in the dark. With tissue which has been fixed in formalin, especially if the period of fixation has been long, there is a tendency toward granular staining. If the tissue has been in formalin very long, the astrocytes may become refractory to the gold.

The following pretreatment has been suggested by Globus for material which has been hardened in formalin. It may be used for both the Cajal and Hortega stains.

- 1 Frozen sections at from 15 to 30 microns
- 2 Wash quickly in several changes of distilled water
- 3 Place in a 10 per cent solution of strong ammonium hydroxide for twenty-four hours at room temperature or for four hours in an incubator
- 4 Rinse rapidly in two changes of distilled water
- 5 Place in a 10 per cent solution of concentrated (41 per cent) hydrobromic acid for two to four hours
- 6 Wash rapidly in two changes of distilled water to which a few drops of ammonium hydroxide have been added. Proceed with the Cajal or Hortega stain as usual.

If sections are not to be stained at once, they should be left in 2 per cent ammonium bromide to which a few drops of neutral formalin have been added. Tissue which has been fixed in formalin ammonium bromide or which has been treated with bromine derivatives may require shorter staining periods in the gold bath. Occasionally, excellent results can be obtained by merely allowing the sections to remain overnight or longer in formalin ammonium bromide in the incubator at 57° C. Penfield suggests the addition of 2 or 3 drops of a 1:1000 aqueous solution of erythrosin to the gold bath to hasten the staining. A similar result can be obtained by adding enough dry erythrosin dye to the gold bath to impart an orange tint. Moderate increases in temperature also favor the staining reaction.

*Corten's Modification* of the Cajal Gold Sublimate stain was devised for formalin fixed tissue and gives good results.

- 1 Frozen sections, 20 to 25 microns, are heated in the following solution until steam arises

Ammonium bromide	15 gm
Formol (undiluted and neutral)	100 cc
Double distilled water	400 cc

Do not boil

- 2 Transfer directly without washing into

Antiformin	3 cc
96% alcohol	8 cc

- 3 Wash thoroughly in two changes of double distilled water



4. Stain for one to two hours or until the sections are brownish red in the following solution:

1% aqueous gold chloride (acid brown)	4 c.c.
5% aqueous mercuric chloride	8 c.c.
Double distilled water	6 c.c.

During the staining, the sections should be kept in the dark in the incubator at 37° C. The staining should be controlled under the microscope but sections should be washed in double distilled water before examination.

5. Wash thoroughly in double distilled water and fix in 5 per cent hypo for fifteen minutes.

6. Wash thoroughly in distilled water, mount on a slide from 50 per cent alcohol, blot firmly but carefully, complete dehydration in absolute alcohol, and clear in xylol. Mount in balsam.

The antiformin should be active and it may be necessary to prepare it fresh. The final results of the stain are those described previously.

Gelatin-embedded material may be used if sections are cut at 10 microns or less. Wertham advises the following gold bath:

1% gold chloride	10 c.c.
5% aqueous mercuric chloride	6 c.c.
Distilled water	4 c.c.

The time required for staining is usually lengthened.

In general, the stain is the simplest and the most reliable of the metallic methods for glial cells.

*Kanzler's Modification of Hortega's Method for Microglia.*—Of the numerous modifications of the original Hortega method for the silver impregnation of microglia, this one gives good results. Tissue fixed in formalin or formalin-ammonium bromide may be used.

1. Frozen sections, 25 to 30 microns, are received in distilled water and transferred to the following formalin-ammonium bromide mixture in which they are heated until steam arises:

Ammonium bromide	15 gm.
Pure formalin	100 c.c.
Distilled water	400 c.c.
This solution keeps well.	

Allow to cool fifteen minutes or longer. Do not boil.

2. Without washing, place directly into the following alcohol-antiformin bath:

Antiformin	3 c.c.
96% alcohol	8 c.c.
Distilled water	2 c.c.
Prepare fresh before using.	

It is essential that the antiformin be fresh and active. Allow to remain five to eight seconds, or until the white matter becomes apparent. Keep sections in motion during this time.

3. Wash in two changes of distilled water and transfer to the following silver bath:

10% silver nitrate	5 c c
10% sodium carbonate	15 c c

Dissolve the resultant precipitate by the addition of strong ammonium hydroxide, drop by drop, shaking continuously. Avoid an excess of ammonia. No further water is added. Filter before use. Prepare fresh.

Allow sections to remain eight to fifteen seconds

4. Transfer without washing to 2 per cent formalin made up with distilled water. Develop quickly, keeping the sections in motion and renewing the solution frequently.

5. Wash freely in distilled water

6. Tone for ten to twenty minutes in the following gold bath.

1% gold chloride (yellow)	1 to 2 c c
Distilled water	10 c c

7. Wash and fix in 10 c.c. of a 5 per cent solution of sodium thiosulfate to which 1 gm. of sodium sulfate has been added. The addition of the latter is not absolutely necessary. Allow sections to remain for one minute.

Wash well in distilled water. Mount sections from 50 per cent alcohol, blot firmly, and complete dehydration in absolute alcohol. Clear in creosote carboxylol and mount in balsam.

Microglia is seen as small cells containing an elongated, rod-shaped or triangular nucleus and short, irregular processes. The cells are stained a grayish black and stand out sharply from the light gray background. There is impregnation of the nuclei of the other interstitial cells, but only rarely can the outline of the astrocytes be seen. Axis cylinders, myelin sheaths, neurons, and such are not stained.

Failure to obtain good staining may be due to several factors. The sodium carbonate used in the preparation of the silver bath must be free from impurities. Imperfectly distilled water may account for some failures. The antiformin must be active, and it may be necessary to prepare it fresh by mixing equal parts of 15 per cent sodium hydroxide and 20 per cent sodium hypochlorite. The commercial antiformin may not give good results. It is often necessary for the individual worker to try minor empirical variations in the technique if the method does not succeed the first few times. The pathologic forms of microglia stain more easily than the microglia in normal brain tissue. The beginner will find it helpful to become acquainted with the method through the use of tissue from a case of dementia paralytica where

the microglia cells are increased in size and number, particularly in the frontal lobes. In general, this method gives fairly consistent results, and the completed preparations are excellent for photography.

*Penfield's Second Modification for Combined Oligodendroglia and Microglia.*—With this modification of *Hortega's* silver carbonate method there is staining of both the oligodendroglia and microglia cells. It can be applied to formalin or formalin-ammonium bromide fixed tissue.

1. Frozen sections, cut at 20 microns, are received into 1 per cent formalin or distilled water. The former is preferable, since it prevents swelling of the tissue.

2. Place sections in distilled water to which 10 to 15 drops of strong ammonium hydroxide have been added. Cover and allow to remain overnight.

3. Without washing, transfer sections to 5 per cent Globus' hydrobromic acid, and place in incubator at 38° C. for one hour.

40% hydrobromic acid	5 c.c.
Distilled water	95 c.c.

4. Rinse in three changes of distilled water.

5. Mordant sections in 5 per cent sodium carbonate for one hour. They may remain here for five to six hours without ill effect.

6. Pass sections, preferably without washing, into *Hortega's* weak ammoniacal silver carbonate solution for three to five minutes, or until they turn a smooth gray when transferred to the reducer. At times good results may be obtained by leaving the sections in the silver solution until they turn light brown. The impregnation should be controlled under the microscope. *Hortega's* weak ammoniacal silver carbonate solution is prepared as follows:

10% silver nitrate	5 c.c.
5% sodium carbonate, pure	20 c.c.

Add sufficient fresh ammonium hydroxide, drop by drop, to just dissolve the precipitate, stirring constantly. Do not add too much ammonia. A fine black precipitate, unlike the voluminous silver carbonate, remains. Filter and dilute to 75 c.c. Keep in a dark bottle.

7. Reduce in 1 per cent formalin, agitating sections constantly. Use fresh formalin for each section.

8. Wash in distilled water and tone in 1:500 gold chloride at room temperature until sections are a smooth gray.

9. Fix in 5 per cent sodium thiosulfate for a few minutes.

10. Wash freely in distilled water and mount on a glass slide from 50 per cent alcohol. Blot, complete dehydration in absolute alcohol, clear with ereosote carboxylol, and mount in balsam.

With this method there is staining of the oligodendroglia and microglia with a fair degree of consistency. The two types of cells can be distinguished by their differences in morphology, the oligodendroglia appearing as rela-

tively small cells with large *spherical nuclei*, scanty cytoplasm, and a few fine processes. The microglia appear as described in the Kanzler stain. Occasionally there is faint impregnation of the astrocytes, especially if the sections have been allowed to remain in the silver bath too long. The background is practically colorless, axis cylinders, neurons, and such not taking the stain. By allowing the sections to remain in the ammonia two nights and lengthening the time of treatment in the silver bath, a more intense impregnation is obtained.

The method works best if the tissue is not too old although good results can be obtained on old formalin fixed material. Tissue fixed in formalin for not over two days gives excellent results. As in the Kanzler modification, failure to obtain good impregnation may be due to imperfectly distilled water, impure sodium carbonate, or improperly fixed tissue. Whenever possible, tissue fixed in formalin ammonium bismide should be used and the silver carbonate solution prepared fresh, although the latter keeps for several weeks when tightly stoppered and stored in an amber bottle in the dark.

*Glia Stains on Paraffin Sections*—Practically all of the selective metallic methods for the demonstration of glia cells require the use of frozen sections. Occasionally, however, unembedded tissue is not available and it becomes necessary to use paraffin sections. Numerous methods have been devised for the silver impregnation of paraffined tissue none of which are very satisfactory. The methods are usually not very reliable and rarely are they selective, impregnating nerve fibers, glia cells, glia fibers and connective tissue at the same time. They are useful, nevertheless, if only as a means of obtaining a general survey for comparison with the more specific stains.

*Wilder's Method for Glia and Nerve Fibers*—Paraffined sections, cut at 8 to 10 microns, are brought to distilled water. Tissue should be fixed in formalin.

- 1 Place in 34 per cent hydrobromic acid for thirty minutes
- 2 Wash in distilled water for ten to twenty seconds
- 3 Flood the slide for five seconds with 1 per cent aqueous uranium nitrate solution, sodium free. It may be necessary to use a shorter time
- 4 Wash in distilled water for ten to twenty seconds
- 5 Place in silver diamino hydroxide for twenty seconds

To 5 cc of 10.2% silver nitrate, add strong ammonium hydroxide until the precipitate which forms just redissolves. Add 5 cc of 31% sodium hydroxide and dissolve the precipitate which forms by adding ammonium hydroxide, drop by drop. Make up to 50 cc with distilled water. Prepare fresh just before use.

- 6 Wash briefly in distilled water
- 7 Agitate each slide separately in the following reducing solution until the brown cloud is no longer given off

1% uranium nitrate	15 cc
40% formalin neutralized with magnesium carbonate	05 cc
Distilled water	500 cc

8. Wash in distilled water.

9. Counterstain with eosin. Dehydrate in alcohol, clear in xylol, and mount in balsam. Hematoxylin may be used but must be blue in tap water.

The ganglion cells, nerve fibers, and glia cells and their processes are stained black. The astrocytes and spongioblasts are well impregnated. Tissue fixed twenty-four hours after death shows impregnation of the nerve fibers and fibrous astrocytes, but the processes of the protoplasmic astrocytes and the oligodendroglia cells cannot be shown when more than six hours have elapsed between the time of death and fixation. As in other metallic methods, if fixation has not been prompt, the staining is apt to be granular.

The uranium nitrate and silver nitrate solutions keep indefinitely. The silver nitrate should be stored in a dark bottle out of the light. The silver diamino-hydroxide, if stored in a tightly stoppered amber-colored bottle, will keep for a week.

*Fincher's Silver Carbonate Method.*—This method is particularly well adapted for tumor tissue. It utilizes Ortega's silver lithium carbonate. Thin paraffin sections are brought to distilled water.

1. Mordant in the following solution at 60° C. until sections are colored yellow:

2% silver nitrate	30 c.c.
Pyridine	10 drops
Alcohol	15 drops

2. Dip in 95 per cent alcohol. Wash sections in water and impregnate in the following silver bath:

Silver carbonate, lithium	30 c.c.
Pyridine	10 drops
Alcohol	15 drops

Place in the incubator at 60° C. until sections are amber.

3. Wash rapidly in 95 per cent alcohol.

4. Reduce in 10 per cent formalin.

5. Wash in distilled water.

6. Tone in 1:500 gold chloride solution in the incubator at 60° C. until sections are a grayish-red color.

7. Wash in distilled water and fix in 5 per cent sodium hyposulfite solution.

8. Wash in distilled water, dehydrate in alcohol, clear in cresote carboxylol, and mount in balsam.

The neural and glial elements are stained a pinkish-red color in contrast to the more heavily impregnated fibroblastic elements which assume a dark brown color. Astrocytes and spongioblasts are well stained.

Zenker fixed tissue may be used but should be passed first through Giam's iodine, followed by 5 per cent sodium thiosulfate to remove the mercuric chloride crystals. Tissue which has been fixed in Kaiserling's fluid for museum purposes may also be stained. An excess of silver in the tissue may be removed by allowing the sections to remain overnight in xylol to which a few drops of pyridine have been added.

Hortega's silver carbonate (lithum) is prepared as follows:

10% silver nitrate	5 cc
0.5% lithium carbonate	20 cc

Add strong ammonium hydroxide drop by drop until the precipitate is just dissolved. The solution should be stirred continuously and care taken to avoid an excess of ammonia. A small amount of fine black precipitate will remain and may be filtered off. Make up to 75 cc with distilled water and store in firmly stoppered brown bottle. The solution keeps for several weeks.

*(To be continued)*

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**TYPHOID VACCINATION**, A Study of Oral, as Measured by Blood Serum Agglutinins, Crimm, P. D., and Short, D. M. *Am. J. M. Sc.* 196: 826, 1938.

Using typhoid-mixed vaccine, 100 cases were vaccinated by the oral and a like number by the subcutaneous method. Blood serum agglutinins were determined periodically.

Thirty-three per cent of the cases which received the oral typhoid-mixed vaccine had mild reactions, none of which were incapacitating.

Significant agglutinin titers with oral typhoid vaccine are present within sixty hours. The agglutinin titration of both methods is essentially comparable after an interval of from two to five weeks. Significant titers persist for six months or longer following oral vaccine.

Those cases failing to show agglutinins five weeks following vaccination were 24 per cent for the subcutaneous, and 8 per cent for the oral cases. Thus, the failures are three times as numerous with the subcutaneous as with the oral method.

The authors conclude, from the extensive clinical reports of others and from the present laboratory results, that the oral method is as efficacious as the subcutaneous method of mixed typhoid vaccination.

**MALARIA**, New Method of Staining Thick Films, Simons, H. *Bull. Soc. path. exot.* 31: 100, 1938.

For the purpose of rapid diagnosis of blood parasites the author advocates the use of the following mixture, which dehemoglobinizes the red blood cells by virtue of the saponin, and stains the parasites and leucocytes by virtue of the methylene blue.

Distilled water	300 c.c.
Methylene blue	0.6 gm.
Sodium chloride	1.8 gm.
Sodium citrate	3.0 gm.
Saponin	2.0 gm.
Formol (15 per cent)	12 c.c.

The mixture is poured over a thin or thick smear and allowed to act for one or two minutes. In the case of thick films the stain is poured off and fresh stain is applied. According to the thickness of the film, this is repeated three to five times. When staining is complete, the stain is poured off and a cover glass is applied, examination of the film being made in the small quantity of stain remaining under the cover glass. There being little danger of the thick film floating off the slide, drying in the first place may be conducted rapidly by repeated gentle warming in a flame and blowing. The whole process of preparation and staining of a thick film does not occupy more than three or four minutes—a considerable saving of time when rapid diagnosis is necessary.

**ERYTHROCYTES**, Red Blood Cell Values in Adolescence, Mugrage, E. R., and Andresen, M. I. *Am. J. Dis. Child.* 56: 997, 1938.

The quantity of hemoglobin, the number of red blood cells, and the volume of packed cells were determined accurately on samples of venous blood from 80 boys and 80 girls, between the ages of 13 and 21 years. The values obtained are compared with a series of 640

similar determinations on the blood of infants, children and adults. From the results of the study, the following conclusions may be drawn:

1. Averages for boys are almost identical with those for girls at 13 years. These gradually increase to the adult level for men at 17 years. Averages for girls show little variation from the level for women during the adolescent period.

2. In the adolescent as well as in the adult the average volume of the individual red blood cell of the male is smaller than that of the female. Values for adolescents of both sexes are slightly higher than those for adults.

3. The corpuscular hemoglobin concentration remains at the same level throughout adolescence as in other age periods.

**DYSENTERY** Specificity of Agglutination Reaction for *S. dysenteriae*, Mackie, T. T.  
Arch. Int. Med. 62: 783, 1938

Prolonged serologic and cultural studies indicate the necessity for cautious interpretation of the agglutination reaction in cases of chronic inflammatory disease of the colon. The author has repeatedly found agglutinins for *S. dysenteriae* present at titers commonly considered to establish the diagnosis unaccompanied by cultural evidence of homologous infection. Conversely, no agglutination reactions have been observed in cases of proved infection. Marked and unaccountable variations of agglutinin titer are the rule in the course of repeated determinations. It is a striking fact that the majority of the sera which gave a high titer were obtained from patients who consistently showed sterile culture for *S. dysenteriae*. These observations suggest that agglutinins for *S. dysenteriae* may develop in response to nonspecific heterologous stimuli.

**LEAD HAZARD**, Observations on Men Exposed to and an Original Method for Detection of Polychromasia, Windsor McLean, L. A. M. J. Australia 2: 367, 1938

Ordinary thin blood films are made on clean thin (10 mm. or less) microscopic slides in the ordinary way. After drying, the films are fixed by running pure methyl alcohol on the slide, film up, and allowing it to remain for two minutes. The film is allowed to dry in the air, and then Sells' stain is run on and allowed to remain on the film for forty-five seconds. The stain is then washed off with running tap water, and the washing continued for one minute. The film should then be very pale green in color. It is allowed to dry in the air and is then examined by the dark field method.

**FREI TEST**, Evaluation of, With Mouse Brain Antigen, Reider, R. F. and Canizares, O.  
Arch. Dermat. & Syph. 38: 628, 1938

For 116 patients with lymphogranuloma venereum and 204 control subjects, 398 tests were made with mouse brain antigen, 398 with emulsion of normal mouse brain, and 124 with antigen prepared from pus of human patients.

Criteria are offered for interpretation of the test with mouse brain antigen.

Of patients with lymphogranuloma venereum, 90 per cent gave positive, 9 per cent negative, and 1 per cent doubtful reactions to mouse brain antigen. Most patients giving a negative reaction had the disease in an early stage and later gave a positive reaction.

Of the control subjects 93 per cent gave negative, 5 per cent positive, and 2 per cent doubtful reactions to mouse brain antigen.

One per cent of persons tested showed a strong hypersensitivity to mouse brain tissue, the reaction to emulsion of normal mouse brain and to mouse brain antigen remaining intense for more than a week.

The optimum time to read the result of the Frei test is between the third and the fifth days, preferably the fourth day.

A reaction to mouse brain antigen on the fourth day consisting of a papule more than 6 mm. in diameter, accompanied with a much less intense reaction to emulsion of normal mouse brain, may be considered positive. The reaction should remain this size or become larger for at least one week.



There is usually a reaction to mouse brain antigen and to emulsion of normal mouse brain during the first two days, which then disappears. This is a nonspecific reaction.

In the interpretation of borderline reactions (6 to 7.5 gm.) to mouse brain antigen, the size of the corresponding reaction to emulsion of normal mouse brain should be taken into consideration. A small percentage of reactions are doubtful and cannot be interpreted. The test should be repeated in cases in which such a reaction appears.

Comparison of mouse brain antigen with that prepared from the pus of human patients shows that both give approximately equal reactions.

Mouse brain antigen is suitable for use in the diagnosis of lymphogranuloma venereum when the reaction to it is properly interpreted.

**TUBERCULOSIS, Effect of, on Serological Reactions for Syphilis, Farran, T., and Emerson, K. Am. Rev. Tubere. 39: 1, 1939.**

Inasmuch as with the present serologic tests for syphilis both typical and atypical false-doubtful and false-positive results are found in sera from tuberculous donors, it is evident that tuberculous toxemia may contribute a confusing factor to syphilis serology. It should not, however, present a major problem in the clinical interpretation of results obtained with carefully conducted serodiagnostic procedures.

**LEUKEMOID REACTIONS of the Myeloid Type, Heck, F. J., and Hall, B. E. J. A. M. A. 112: 95, 1939.**

The importance of differentiating leucemoid reactions from the leucemias is obvious. In most cases this differentiation can be made on the basis of the clinical observations. The hematologic picture may or may not be of additional aid in diagnosis. The total leucocyte count is of relatively little or no diagnostic significance unless it is in excess of 100,000 cells per c.mm. blood. Leucocyte counts above this are observed rarely with conditions other than the chronic forms of leucemia. It should be remembered that many patients with leucemia, including the chronic variety, have total leucocyte counts which fall either within or below the range for normal persons. In a study previously reported, approximately 40 per cent of all patients with acute leucemia and 10 per cent of all patients with chronic leucemia seen at the Mayo Clinic from 1928 to 1933, inclusive, had leucocyte count below 10,000 cells per c.mm.

*Summary.*—Leucemoid reactions occur commonly with infections, with blood dyscrasias and diseases of the reticulo-endothelial system, with diseases in which there is invasion and irritation of the bone marrow (as in metastasis to bone), with conditions in which there is an increased demand on the bone marrow (such as severe and sudden loss of blood), and with chemical poisoning. Occasionally cases are encountered in which no satisfactory explanation for the leucemoid reaction can be made.

**TISSUE: Preparation of Plaster of Paris Embedding Boxes, Solberg, A. N. Stain Technol. 14: 27, 1939.**

Cut pieces of paraffin of the size you desire the blocks. Bevel the paraffin block about 15° so that the bottom will be smaller than the top to allow the paraffin blocks to slip out when shrunk by cooling. Seal the block, wide side down, onto a copper plate or piece of cardboard, and place around it two embedding angles about 1/8 inch from the widest part of the paraffin block. Coat the paraffin block with a thin layer of vaseline. Mix enough plaster of Paris and water to fill the mold, and pour the mixture into the mold while still very wet. Allow the plaster of Paris to dry for an hour or more. Remove the embedding angles, and when the plaster of Paris seems quite dry, lift the box off the paraffin mold. When dry, trim the box with a knife and sandpaper to the desired shape and thickness. It is advisable to leave the wall quite thick to prevent breakage.

To use the box: Soak it for a moment in the water to be used for cooling. Pick it out and pour out the water. Holding the box between the thumb and forefinger, fill the box with melted paraffin using a medicine dropper. Drop the tissue into the melted paraffin.

Orient tissue as desired with a warm needle or forceps. Insert a previously prepared slip of paper with identification marks on it into the melted paraffin at one end of the box. Hold the box in the cold water to cool and blow gently on the surface until a thin film has congealed. Carefully lower the box under water and in a few seconds the paraffin will shrink from the sides of the box, allowing the block to float free. The box may be used again immediately, but one can work faster by using two boxes alternately.

**HYPERGLYCEMIA, A New Interpretation of, in Obese Middle Aged Persons** Newburgh, L. H., and Conn, J. W. J. A. M. A. 112, 7, 1939

A statistical analysis of spontaneous glycosuria associated with delayed disposal of ingested dextrose indicates that approximately half of the patients are obese. These obese glycosuric patients are, with few exceptions, more than 30 years of age.

After the weight of these patients has been reduced to normal by simple underfeeding, they remain aglycosuric, do not become hyperglycemic when they are placed on maintenance diets containing 300 gm. of carbohydrate, and exhibit normal dextrose tolerance curves.

There is an occasional exception to this rule, but more than 90 per cent of the patients respond in this manner.

Recurrence of the obesity is capable of reproducing the hyperglycemia and the delayed utilization of dextrose. Subsequent reduction of weight again corrects the disturbance in the metabolism of carbohydrate.

It has been demonstrated that the majority of persons who have been obese for many years show delayed utilization of carbohydrate. Since the majority respond in this way to adiposity and again dispose of carbohydrate normally when the excessive weight has been removed, this phenomenon must be regarded as being the type response of the previously normal mechanism carbohydrate metabolism to the overload of obesity.

It is suggested that the occurrence of the hyperglycemia and glycosuria in such persons depends on the excessive accumulation of fat in the liver, with a resulting impairment in its capacity to lay down glycogen at the normally rapid rate.

The studies described establish a clinical entity in which obesity is the principal abnormality and hyperglycemia is a secondary phenomenon.

**AGGLUTINATION, Salt Optima in, Platt, A. E.** Australian J. Exper. Biol. & M. Sc. 16, 275, 1938

Using a smooth strain of *Brucella abortus* and a corresponding antiserum obtained from an infected cow, it was found that the antigen and antibody optimal ratios remained constant for all salt concentrations between 2M/7 and M/56. Concentrations of salt above 2M/7 were not used, and those below M/56 failed to agglutinate the sensitized organisms.

The optimal salt concentration for any agglutinating system depends upon (a) the ratio of antigen to antibody, (b) the concentration of organisms per unit volume of mixture, and (c) the effect of the salt on the cohesive force between the organisms.

**GOLD SOLS, Variations in the Behavior of Paretic Cerebrospinal Fluids With Different Types of, Pennycuik, S. W., Woolcock, C. E., and Cowan, R. J.** Australian J. Exper. Biol. & M. Sc. 16, 317, 1938

The oxalate reduced gold sol can be used in the Lange test if a buffering agent is added to the solution either before or after the reduction of the gold salt. In general, however, it is not as reliable as the formaldehyde reduced sol.

The phosphorus reduced gold sol cannot be used with any degree of precision.

The Bredig gold sol can be adapted for use, but its reactions are variable and its preparation is inconvenient.

A trace of stannic chloride when added to distilled water facilitates the formation of gold sols, but such sols, while they are suitable for paretic cerebrospinal fluids, show slight irregularities with normal fluids.

**PREGNANCY, Regulation of Level of Calcium in the Serum During,** Bodansky, M., and Duff, V. B. J. A. M. A. 112: 223, 1939.

Within certain limits (not lower than 8.5 mg.) the depression of calcium in the serum observed in late pregnancy may be considered a normal condition, conducive perhaps to a greater economy of utilization of calcium derived from food and maternal bodily reserves.

Severe hypocalcemia during pregnancy may reflect either parathyroid deficiency or marked nutritional deficiency. Both are comparatively rare in ordinary clinical experience. The importance of the parathyroid glands in the regulation of the calcium content becomes especially impressive in the parathyroid-deficient pregnant animal at term.

Abnormalities in fetal skeletal development occur if hypocalcemia is severe, as shown by the results in experimental parathyroid deficiency and in clinical osteomalacia. The somewhat elevated calcium level in the fetal circulation may, therefore, be looked upon as normal for fetal development.

Nutritional, hormonal, and seasonal factors influence the maternal calcium level. The influence of season may be only partly related to the amount of available sunshine.

It is possible that in pregnancy the maintenance of a subnormal calcium level in the maternal serum, as judged by nonpregnancy standards, despite the augmentation in parathyroid function, is dependent on an intrinsic calcium-depressing factor. The maintenance of a relative elevation of calcium in the fetal serum may also indicate the participation of some factor in the fetal organism independent of the fetal parathyroid glands. The rise in the maternal calcium level after parturition and the decline in that of the newborn infant suggest that these factors may reside in the maternal and fetal portions of the placenta, respectively, each playing a distinct and important role in the regulation of the maternal and the fetal calcium levels. The decline in calcium content observed in the newborn may also be conditioned by the sudden withdrawal of the labile calcium reserve of the fetal placenta.

**MENINGOCOCCIC INFECTIONS, Sulfanilamide in the Treatment of,** Waghelstein, J. M. J. A. M. A. 111: 2172, 1938.

There were 106 patients with meningococcal infection treated at Sydenham Hospital with 19 deaths, a fatality rate of 18 per cent. The case fatality rate in the adequately treated group was 12 per cent.

When sulfanilamide alone was used, the fatality rate was 15 per cent. In the adequately treated cases, the fatality rate was 12 per cent.

The combined method of treatment was used in 34 cases, with a case fatality rate of 24 per cent. The fatality rate in the adequately treated group was 13 per cent. Among the last 26 patients treated in this manner there were 3 deaths, 2 of which occurred within twenty-four hours.

The spinal fluid cultures were more consistently sterile after from twelve to twenty-four hours of treatment in those patients in which sulfanilamide was taken every four hours by mouth or by nasal catheter, rather than when given every twelve hours by hypodermoclysis.

The number of lumbar punctures in the treatment of meningitis has been markedly reduced.

The complications due to sulfanilamide therapy were infrequent and very mild. The number of recurrences were reduced. Those complications due to meningococcal infections were not significantly reduced in occurrence.

Since the introduction of sulfanilamide in the treatment of meningococcal infection, there has been a definite decrease in the number of deaths, not only in the adequately treated, but also in those fulminating cases which usually terminate fatally within twenty-four hours. The discomfort suffered by the patient directly due to the treatment is lessened owing to the simplification of the treatment, and the hospital stay has been notably reduced.

# *The Journal of Laboratory and Clinical Medicine*

VOL 24

JUNE, 1939

No 9

## *CLINICAL AND EXPERIMENTAL*

### A NONSTATISTICAL EVALUATION OF ARTIFICIAL PNEUMOTHORAX\*

PAUL H. RINGER, A B, M D, F A C P ASHVILLE N C

**I**N THE past twenty five years artificial pneumothorax has grown from being a method of collapsing an extensively diseased tuberculous lung in a definitely unilateral case, to a procedure advocated by some in minimal cases of tuberculosis, a procedure used diagnostically in cases of suspected lung tumor, a procedure used therapeutically in lobar pneumonia, a procedure advocated in massive atelectasis, a procedure often employed bilaterally, a procedure utilized in cases that almost from the start became ambulatory, a procedure of constantly increasing importance, and one which has a definite bearing on public health, a procedure deemed so necessary that in many states rural practitioners are given instructions in refills at the state sanatorium, and they return to their homes supposedly qualified to continue injections upon their patients discharged from the institution. It is well to pause for a few minutes to evaluate this method, the "ancient of days" among the means employed in collapse therapy.

It is a fair cry from James Carson of Liverpool who, in 1821, first advocated pneumothorax, to our present day handling of the matter with pneumothorax clinics, attempts at collapse of all cases, etc. Unquestionably, successful artificial pneumothorax is the simplest and most effective method for closing cavities, controlling toxemia, and improving the ultimate prognosis. Cutler<sup>1</sup> has shown that the patient with moderately advanced tuberculosis treated with pneumothorax has a chance for recovery six times as great as the patient treated without pneumothorax. Huby<sup>2</sup> has emphasized the importance of pneumothorax from a public health standpoint in the diminution of the source of infection by converting open cases into closed ones, and in the shortening of

\*Read before the meeting of the American Clinical and Climatological Association in Baltimore Md., October 11, 1937.

Received for publication September 15, 1938

sanatorium residence, thus speeding sanatorium turnover—a very necessary factor in health economics.

An evaluation of this important procedure should be made upon the premises of indications, management, complications, and termination.

I certainly would not dwell upon the run-of-mine indications for pneumothorax, other than to say that primarily it is induced to close cavities and to render sputum negative. It seems to me, however, that not infrequently a mistake is made in paying too much attention to *extent* of pathology and not enough to *type* of pathology; in other words, the benign exudative and the caseous pneumonic types. Ulmar<sup>3</sup> notes that the benign exudative type undergoes spontaneous resolution, while the caseous pneumonic type, as the name implies, caseates and later cavitates. After the cavity has developed, it is time to collapse, for obviously no influence can be exerted upon a solid lung, as it is axiomatic that the only lung that can be collapsed is an air-containing lung, which the pneumonic lung is not. On the other hand, Eglee and Jones<sup>4</sup> warn against the diagnosis of the benign form of exudative type of tuberculosis, believing that there is no reliable diagnostic criterion and that all forms of pulmonary phthisis are malignant until they prove themselves to be otherwise. They also believe that the acute pneumonic type should be collapsed, feeling that there is much to gain from an immediate pneumothorax and all may be lost by delay. Hence here are two diametrically opposed views on the question.

There are seen nowadays more patients with acute bronchopneumonic onset than in the past. Why this is true, I do not know, unless, perhaps, that with a total population immunity increasing, those not effectively protected react more violently to the infection. I feel that it is advisable to try to use collapse therapy on these patients at once, though one is tempted to wait because of the acuteness of the toxemia, but some think that it may be better to wait awhile, if possible, until cavitation begins, despite the fact that while waiting one must necessarily run the risk of a bronchiogenic spread. The time when collapse should be started in this type of tuberculosis involves nicety of judgment based upon experience, and in every case it should be induced very slowly.

Collapse of minimal lesions and ambulatory pneumothorax strike me as being "second best" methods. Pneumothorax is fraught with certain dangers known to all of us. Why subject the truly minimal patient to them unless the pathologic process shows a tendency to spread, in which case the patient no longer belongs to the minimal class? I am fully aware of the arguments of O'Brien, of Detroit, in favor of minimal collapse, but I cannot agree with them. He states: "While it is recognized that patients with tuberculosis go through various phases, there is none in which collapse therapy is not indicated except in the terminal stage where it is too late."

The minimal patients are those that should and will recover under bed rest alone. J. A. Myers<sup>5</sup> recommends it on the following grounds: 1. Clinical results are equal to those obtained with other methods. 2. The patient is not immediately condemned to prolonged and costly inactivity. 3. Both the community and the patient save money. Cole and Harper<sup>6</sup> reach practically the same conclusion. I submit, however, that both authorities are working in

clinics in large cities, serving a native population predominantly of the lower classes and that, therefore, from the economic standpoint some compromise measure must be reached. Instead of physical plus physiologic rest, which unquestionably is the best, physiologic rest alone must be relied upon. This situation is not present among patients able to seek a health resort for their cure. It has ever been advocated that pneumothorax is essentially an adjunct and an adjuvant to general physical rest. When both are not to be obtained, certainly the physiologic rest given by pneumothorax is the one to select.

Wilhelm Neumann<sup>7</sup> states that the best results are obtained if pneumothorax is combined with the use of tuberculin and that in most cases healing of cavity takes place in from one and one half to two years, while with pneumothorax alone it may not be complete after from three to four years. This is mentioned simply to preface the statement that while Neumann is recognized as a great authority, his experience can in no way be assented to in this country.

Pneumothorax in lobar pneumonia has its proponents and opponents. Moonman, of Oklahoma City, is in favor of its use. Crowell of Lincoln, North Carolina, has used it on a dozen patients with gratifying results. Behrend<sup>8</sup> and his associates used it on 40 patients and found that pain and cyanosis were relieved and that dyspnea usually disappeared. They also found that the average duration of temperature after pneumothorax was three and one half days, in contrast to seven days when pneumothorax was not used. Hines and Bennett,<sup>9</sup> who treated 12 patients, 4 of whom died (mortality 33 $\frac{1}{3}$  per cent), stress the relief of pain and dyspnea. Bullock,<sup>10</sup> in an extended evaluation of the procedure, is little in favor of it, and wisely comments that pneumothorax has no effect upon the immunity mechanism and does not prevent or cure bacteremia. He feels that its use should never take precedence over the other modern methods of dealing with pneumonia, and therein I heartily concur. My own experience has been limited to two patients, in whom definite diminution of pain and dyspnea were noted, but no change in the course of the disease could be ascribed to pneumothorax.

I must, of necessity, deal very briefly with management and, therefore, am forced to take refuge in "impressions," which the late Dr. Lawson Brown once said "no longer have any place in scientific medicine." Nonetheless, I am convinced, after twenty six years of experience with pneumothorax, that each case is thoroughly individual. Fifteen and more years ago the routine of refills in my practice was fairly well standardized. Today variations in time between injections, as well as the amount of air given, are practically coequal with the number of patients under treatment. I do not know the reason, but I feel that it rests upon one of two factors: either the present day tuberculous patient is exhibiting a different type of reaction to his infection (which I suspect) or else with added experience, better judgment is being exercised in the administration of pneumothorax.

Bilateral partial pneumothorax, unthought of or deemed impossible in the early days, has become relatively common (as has its child, bilateral partial thoracoplasty). I know of no pulmonary therapeutic procedure necessitating more sapient judgment than the adoption of this method. In wise hands

wonderful results are obtainable, but it is a procedure that should be restricted to the specialist in the treatment of pulmonary tuberculosis. As foster father to bilateral pneumothorax, may be cited the patients with bilateral cavitation; then must be determined the primary point of attack, whether to the right or to the left, which places considerable responsibility upon the physician. While we often employ bilateral pneumothorax when, after a successful collapse of one lung, an acute flare-up takes place in the contralateral lung, bilateral cavitation at the outset is no contraindication to the use of the procedure. As a matter of fact, as pointed out by Pruvost, Leblanc, and Petrand,<sup>11</sup> contralateral cavities are often benefited at first when the mediastinum is mobile—purely on a mechanical basis. One question, however, whether these cavities would ever close with sole reliance on contralateral pressure mechanism.

The question has arisen whether pneumothorax should be induced after the aspiration of fluid resulting from a pleurisy with effusion. I think that the decision reached should depend upon the pathology in the underlying lung and, therefore, should depend upon whether the effusion was idiopathic or a complication of previously known pulmonary disease. If the former, I feel that the lung should be allowed to re-expand under careful roentgenologic observation, until the type of pathology becomes manifest, when the indication for pneumothorax will be clear; if the latter, it depends wholly upon the pre-existing pulmonary pathology.

In any general evaluation of pneumothorax, we must consider the possible complications. I will pass over the hair-raising, dramatic occurrence of air embolism and pleural shock. They are rare and are not forgotten by one who has seen them, but their possibility by no means constitutes a contraindication to the use of the method. Spontaneous pneumothorax superimposed upon an existing artificial pneumothorax is one of the chances which every patient must run. Mobile mediastinum, wherein the further collapse of a partially collapsed lung results only in a shift en masse of the lung and mediastinum toward the sound side with consequent squeezing of the contralateral lung, is mentioned only to be included.

The real complication is *fluid* in all its ramifications from the benign serous exudate of an ounce or so, discoverable only fluoroscopically, to the massive tuberculous empyema, terminated successfully only by thoracoplasty, or unsuccessfully by the development of toxemia, a pleurobronchial fistula, and eventually by death.

Inasmuch as from 50 to 70 per cent of pneumothorax patients develop fluid, it becomes a serious consideration, for, once it has appeared, no one can tell what course it will pursue. The cause of fluid has as yet to be definitely determined and explained. It has been believed by some to be due to the use of high pressure in pneumothorax, but this hypothesis is made negative by fluid occurring in countless cases in which high pressures have never been employed. Ulmar thinks that it is due "to the stimulation of the pleura by some antigenic material." He feels that in pneumothorax on a person who has had a previous infection with the tubercle bacillus, all tissues are sensitive to tuberculous antigen and the pleural membrane, like the peritoneal membrane, is characterized by its ability to pour forth large amounts of fluid when stimulated or irritated.

Serous fluid per se does no harm. Infected fluid harboring tubercle bacilli, is a serious matter. Its origin unquestionably is endogenous, though pathways of mobilization are open to discussion. Tuberculous empyemas can and do clear up spontaneously. In the past five years I know of two cases in my own practice that have done so, though I am convinced that this was due more to good luck than to good management. Nonetheless the appearance of tubercle bacilli in the fluid of the pleural cavity must be viewed with great concern. This concern naturally must be heightened if systemically and bacteriologically evidence of mixed infection is demonstrable.

How long should a successful pneumothorax be maintained? Opinions differ. The thesis has been put forth that a year or eighteen months is ample. Some advocate three, some five, years, others more conservative still, believe in continuing pneumothorax as long as possible basing their contention on the theory that eventually all pneumothoraces will be self obliterating. It seems to me that the period of maintenance should be predicated upon the pathologic picture existing at the time the pneumothorax was instituted. If extensive with large cavity formation, certainly a period of five years is not too long, if relatively slight, with a small cavity or no demonstrable cavity but merely positive sputum, from two and one half to three years should be sufficient. In patients with many adhesions, but in whom a partial collapse has given excellent clinical results, indefinite continuation is indicated. It must be remembered that while the zones of infiltration distant from, as well as adjacent to the cavity may well heal in a year or so, it takes a far longer time for the reopated walls of the cavity to become permanently adherent and furthermore, that there is no test that we can apply as a result of which we can be certain that when pressure is released and the resultant *vis a tergo* comes into play, they will not separate and "the last state will be worse than the first."

As pointed out by Dundee," age and occupation have their places in the program of re expansion. The younger the patient, the more time should be given to collapse for relapse is more frequent in the younger age groups. Also, if the patient, when again taking his place in the world, is obliged to do hard, manual labor, collapse should be continued over a longer period of time.

This question of duration of collapse is a vexatious one from the standpoint of both the physician and the patient, from that of the former, who feels that the final proof of a successful pneumothorax is shown in the deliberate allowance of the lung to re expand, and from that of the latter who usually having feared the initial injection with mortal terror, now feels that if his lung is permitted to re inflate, he is thereby cutting the last cable that binds him to the shore of health. The reactions of these patients to the beginning and end of their treatment show that pneumothorax also entails a psychological factor which cannot be disregarded. I have had far more heated arguments with patients about discontinuing pneumothorax than about initiating it. To the patient, re expansion of the lung signifies a *restitutio ad integrum* and forgetfulness of all that went before. To the pathologist, it means calcification, fibrosis, thickening, contraction, deformity and, perchance, healing. To the physician, it imposes increasing watchfulness lest that which was believed to be



whole shall presently crack and, instead of becoming a monument to his prowess and judgment, will instead develop into an apologia for his human weakness and consequent mistakes.

#### SUMMARY AND CONCLUSIONS

I have attempted very briefly to give an evaluation of artificial pneumothorax. From this report the following conclusions can be made:

Pneumothorax is unquestionably worth while.

Complications resulting from pneumothorax may be serious, sometimes fatal, but only in such a small percentage of cases that their occurrence should not interfere with advising its induction.

Ambulatory pneumothorax as a substitute for the best method of treatment is desirable.

There is no definite rule to follow in giving pneumothorax.

Those who have employed pneumothorax would never voluntarily revert to the precollapse period.

#### REFERENCES

1. Cutler, J. W.: Increasing Importance of Pneumothorax Therapy in Pulmonary Tuberculosis, *J. A. M. A.* 106: 1366, 1936.
2. Hruby, Allan J.: Public Health Aspect of Pneumothorax Therapy, *Illinois M. J.* 60: 367, 1931.
3. Ulmar, David: Pneumothorax: Indications, Mechanism and Management, *Quart. Bull., Sea View Hospital* 2: 307, 1937.
4. Eglee, E. P., and Jones, O. A.: A Review of 100 Re-expanded Cases of Artificial Pneumothorax, *Am. Rev. Tuberc.* 35: 500, 1937.
5. Myers, J. A.: Artificial Pneumothorax: With Particular Reference to Ambulatory Patient, *J. Thoracic Surg.* 6: 513, 1937.
6. Cole, Dean, and Harper, Edgar: *Nat. Tuberc. A. Tr.* 1935.
7. Neumann, Wilhelm: Quoted by Ulmar, *Wien. med. Wchnschr.*, Mar. 26, 1936, and Apr. 4, 1936.
8. Belrend, A., Tuck, V. L., and Robertson, W. E.: Artificial Pneumothorax in Treatment of Lobar Pneumonia. A Review of 40 Pneumothorax-Treated Patients and One Hundred Patients Treated by Other Methods, *J. Lab. & Clin. Med.* 20: 914, 1935.
9. Hines, L. E., and Bennett, D.: Artificial Pneumothorax in Treatment of Acute Lobar Pneumonia, *Arch. Int. Med.* 55: 100, 1935.
10. Bullowa, J. G. M.: Pneumothorax in Pneumonia; An Appraisal, *New York State J. Med.* 35: 1001, 1935.
11. Pruvost, P., Leblanc, M., and Pétraud, M.: Les cavernes contralatérales constitutives une contre-indication au pneumothorax artificiel? *Arch. méd.-chir. de l'app. respir.* 11: 141, 1936.
12. Dundee, J. C.: When Should Refills Be Stopped? *Brit. J. Tuberc.* 30: 55, 1936.

# HEMATOLOGIC INDICES IN NORMAL AND ANEMIC PATIENTS\*

## A TRIAXIAL GRAPHIC METHOD

KATSUJI KATO, PH D M D, CHICAGO ILL

THE triaxial correlation chart recently devised by the writer<sup>1</sup> offers an accurate and convenient method of classifying various types of anemia with reference to their index characteristics. The construction of this chart is based on the logarithmic interrelationship existing between the three major hematologic indices of diagnostic importance namely the color, volume, and saturation indices, respectively. As expressed in index numbers the hematologic attributes of any given sample of blood can be precisely determined by the coordinate position occupied by that particular blood on the chart. A biaxial chart of Haden<sup>2</sup> expresses the same fundamental interrelationship between the color and volume indices and for practical purposes is quite adequate, but the triaxial coordination possesses distinct advantage over the biaxial in that when the other two indices are known the saturation index is automatically registered on the triaxial chart without further calculation. This chart is again quite different from the logarithmic calculation chart of Osgood<sup>3</sup> in that the latter is intended only as a device for the calculation of index numbers.

The object of this paper is to demonstrate the practical application of the triaxial chart as an aid in the diagnosis and treatment of various types of anemia. For this purpose hematologic data from both normal and anemic infants and children have been utilized, together with a certain amount of data in diseases which occur exclusively in adult subjects. The bulk of the data presented is from personal observations, although some has been taken from the literature or supplied to the writer in personal communications by other observers.

Of the methods employed in the present series of study it may be mentioned that in the determination of packed cell volume a specially devised combination microhemopipette (Kato<sup>4</sup>) has been used throughout. This pipette makes use of 0.05 cc of oxalated blood, the double oxalate mixture of Heller and Paul<sup>5</sup> having been used in the proportion of 1:500, thus 0.05 cc of blood used contains 0.1 mg of double oxalate mixture in dry form. All values have been recorded in percentage figures by direct reading of the packed cell volume on etched graduation markings. No attempt has been made to calculate the percentage of correction factors since all determinations were made under

\*From the Department of Pediatrics University of Chicago  
Read before the Section on Pediatrics at the Eighty Ninth Session of the American Medical Association San Francisco Calif June 17 1938  
Received for publication October 12 1938

identical conditions. The red blood cell count was made by the usual hemocytometer method, and the hemoglobin values estimated by the acid hematin method of Newcomer.

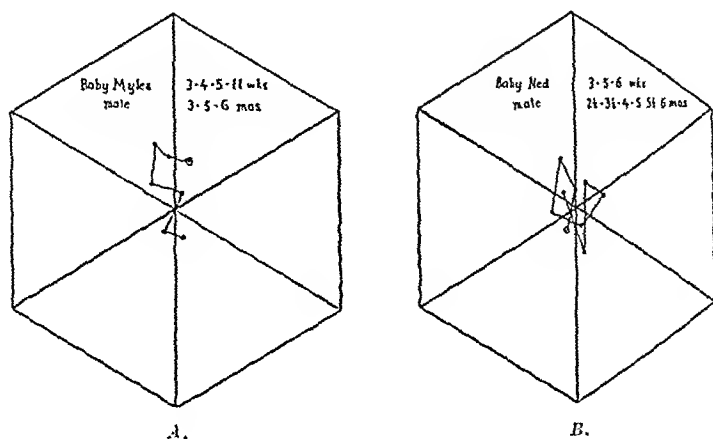


Fig. 1.

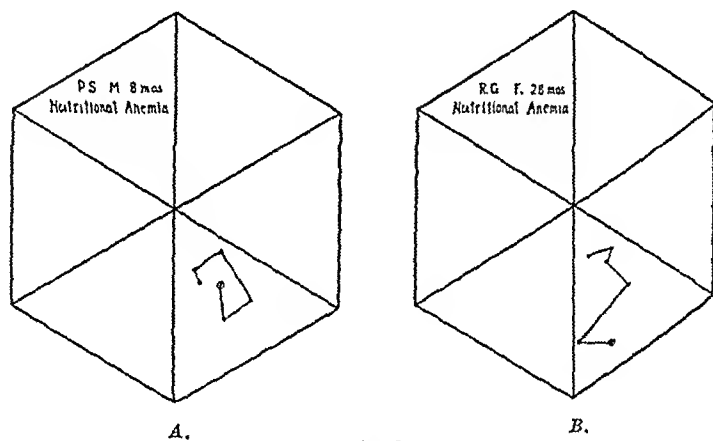


Fig. 2.

The data may be classified according to the following groups, which are presented on triaxial charts as illustrated:

(1) Normal blood of growing infants (2 cases) was observed once a week beginning at the age of three weeks and extending to six months (Fig. 1A and B). For the sake of simplicity only a selected number of observations are actually reproduced. In one of these infants (Baby Myles) the hematologic indices during the first eleven weeks of postnatal life lay outside the range of normal variations, while all subsequent observations were confined within the normal area. In the second baby (Baby Ned) the positions of the indices moved practically within and around the normal zone.

(2) Nutritional anemia in infants is, according to its index position, microcytic, hypochromic, and hypoplenic in nature. The two representative cases are recorded, each showing the movement of its index position under iron therapy toward the center (Fig. 2A and B).

(3) Anemia accompanying infections may be either microcytic or macrocytic, but is usually hypochromic and hypoplenic. In a case of severe rheu-

matie heart disease in a 10 year old female child macrocytosis was constant throughout the period of observation, while in an 11 month old infant suffering from cervical adenitis, the anemia was definitely microcytic, hypochromic, and hypoplenic, signifying this to be a case of superimposition of nutritional anemia (Fig 3A and B). In the latter instance treatment with ferrous sulfate produced a definitely beneficial effect not only in the index positions of the blood but also in general clinical conditions.

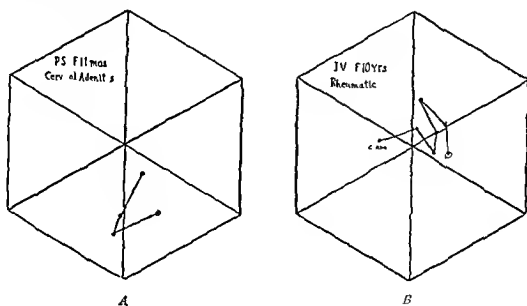


Fig 3

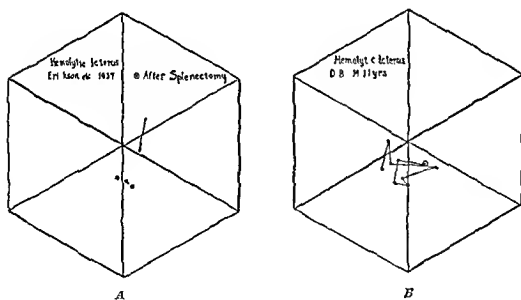


Fig 4

(4) Hemolytic icterus, according to the data given by Erickson and his co-workers,<sup>6</sup> has rather heterogeneous index pictures, the majority tending toward hypochromia and microcytosis (Fig 4A). In one case of mild hemolytic jaundice in a 10 year old male child the index characteristics revealed microcytosis and, for the most part, hypochromia, but with rather constant hyperplenia. In other words, anemia developing in congenital hemolytic icterus is microcytic, hypochromic, and hyperplenic, rather than microcytic and hyperchromic, as it is usually described. This means that in hemolytic icterus the tendency is toward oversaturation of red blood cells with hemoglobin, although the color index remains constantly below normal (Fig 4B).

(5) In those cases in which index numbers are obtainable (Erickson and associates,<sup>6</sup> Bradford and Dye<sup>7</sup>) erythroblastic anemia is characterized by microcytosis and hypochromia of red blood cells (Fig. 5A). Morphologically, however, a number of instances of this anemia are reported to be macrocytic.

(6) Sickle-cell anemia (Erickson and associates<sup>6</sup>) involves practically every type of anemia, as demonstrated by the rather universal distribution of index numbers (Fig. 5B).

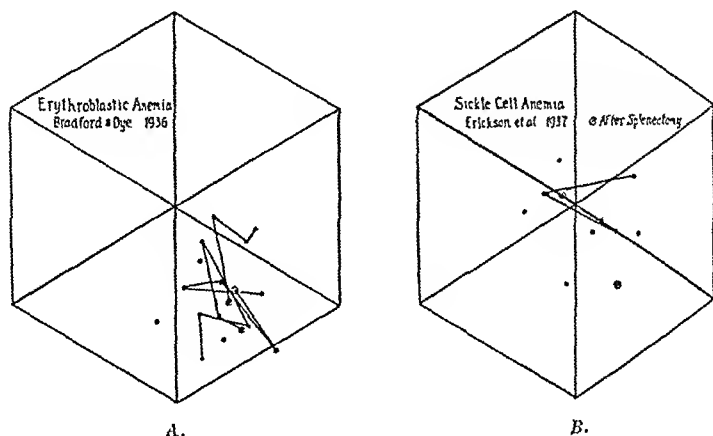


Fig. 5.

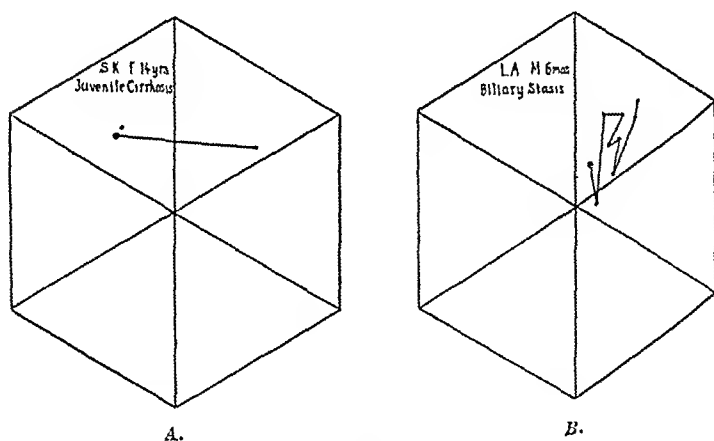


Fig. 6.

(7) Anemia associated with liver diseases tends to be typified by macrocytosis and hyperchromia. In one case of juvenile cirrhosis of the liver in a 14-year-old female child, the initial index position was that of pronounced macrocytosis, hyperchromia, and hyperplenia (Fig. 6A), while in a series of observations of liver cirrhosis in a 6-month-old infant, the index positions fell consistently in the zone of macrocytosis, hypochromia, and hypoplenia (Fig. 6B).

(8) Anemia occurring in mucinaria, of which 2 cases in young children have recently been studied by Buckman,<sup>8</sup> is macrocytic and hyperchromic (Fig. 7A). It is interesting to note that under treatment with liver extract in combination with an anthelmintic, one of these patients showed a decided improvement in hematologic values but developed a pronounced hyperplenia.

(9) In a case of aplastic anemia accompanied by profuse hemorrhage following dental extractions the patient exhibited definite macrocytosis and hyperchromia, in spite of extreme oligocythemia (below 500 000), hypohemoglobinemia (17 gm per cent), and low cell volume (5 per cent). With repeated blood transfusions all hematologic values rapidly increased, but the index numbers moved toward the zone of microcytosis and hypochromia (Fig 7B).

(10) In the majority of cases of pernicious anemia, according to the data published by Williams and his co-workers,<sup>9</sup> the initial index positions are pronouncedly macrocytic and hyperchromic and under liver treatment move toward the normal area (Fig 8A).

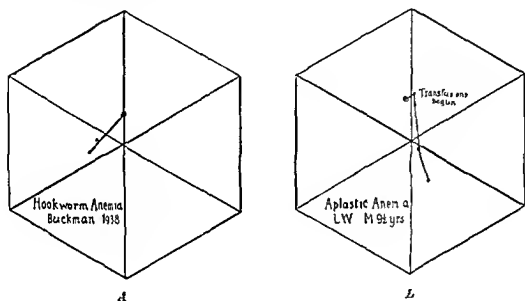


FIG 7

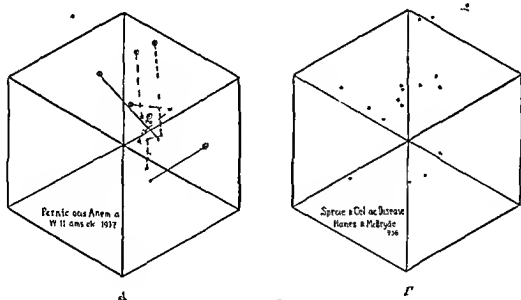


FIG 8

(11) The anemia of sprue and celiac affections, according to the data supplied by McBryde<sup>10</sup> from the cases published by Hanes and McBryde,<sup>11</sup> varies greatly as to index positions. The 17 cases of this group of diseases are predominantly macrocytic and hyperchromic as well as hyperplenic, microcytosis and hypochromia here occurs only in approximately 25 per cent of the cases (Fig 8B). In fact, according to the observations of Thaysen,<sup>12</sup> the anemia in sprue may be either simple hypochromic or hyperchromic, or the picture may even fluctuate back and forth between these two types, depending upon the period of remissions or relapse.

## COMMENT

The hematologic data, as presented on the triaxial index chart, are self-explanatory and are offered primarily to illustrate the concrete manner in which such data can best be recorded. Too frequently the diagnosis of anemia is based solely on a decrease in red blood cell count and hemoglobin value, very little attempt being made in clinical practice to determine the precise nature of the anemia. Use of the triaxial chart makes possible an accurate diagnosis of anemia according to the graphic classification proposed. Quantitative aspects of anemia, however, need always to be correlated with qualitative and morphologic changes of the blood cells, as demonstrated by well-prepared smears. When properly correlated, classification of anemias by the triaxial graphic method is a distinct contribution to exact hematologic diagnosis.

For convenience in recording the triaxial hematologic data either a special hospital record sheet with the index chart printed thereon, or a suitable sized rubber stamp of the chart, might be employed and included in the patient's history. A consistent use of this method for hematologic diagnosis requires complete routine erythrocyte examination, consisting of cell count and accurate hemoglobin estimation, together with packed cell determination.

## SUMMARY AND CONCLUSIONS

An attempt has been made to emphasize the importance of making accurate diagnosis of anemias on the basis of both quantitative and qualitative characteristics of the blood. For this purpose the triaxial index chart, as devised by the author, is offered as a means of enabling clinicians and laboratory workers to make precise classification of anemias.

The concrete manner in which the triaxial index chart is used for this purpose has been illustrated by the nature of index positions in various types of anemias.

Accuracy in the classification of anemias is essential in instituting the specific therapy indicated for each type, and the course of recovery from anemia can be clearly recorded on the chart here advocated.

The triaxial correlation chart for hematologic indices is recommended for general and routine use in clinical and laboratory work.

## REFERENCES

1. Kato, K.: Triaxial Correlation of Hematologic Indices: Its Significance in Classification and Treatment of Anemias, *J. LAB. & CLIN. MED.* 24: 191, 1938.
2. Haden, R. L.: Clinical Factors in the Production of Anemia and the Regeneration of Erythrocytes and Hemoglobin, *M. Clin. North America* 17: 887, 1934.
3. Osgood, E. E.: Tables for Calculation of Color Index, Volume Index and Saturation Index of Different Anticoagulants, *J. LAB. & CLIN. MED.* 12: 899, 1927.
4. Kato, K.: Combination Microhemopipette, *J. LAB. & CLIN. MED.* 23: 980, 1938.
5. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, *J. LAB. & CLIN. MED.* 19: 777, 1934.
6. Erickson, B. N., Williams, H. H., Hummel, F. C., Lee, P., and Macy, I. G.: The Lipid and Mineral Distribution of the Serum and Erythrocytes in the Hemolytic and Hypochromic Anemias of Childhood, *J. Biol. Chem.* 118: 569, 1937.

- 7 Bradford, W L, and Dye, J Observations on the Morphology of the Erythrocytes in Mediterranean Disease—Thalassemia *J Pediat* 9 312 1936
- 8 Personal communication from Dr Thomas L Buckman Jacksonville Fla
- 9 Williams, H H, Erickson, B N, Bernstein S, Hummel, F C and May, I G The Lipid and Mineral Distribution of the Serum and Erythrocytes in Pernicious Anemia, *J Biol Chem* 118 599, 1937
- 10 McBryde, A Personal communication
- 11 Hanes, S M, and McBryde, A Identity of Sprue Nontropical Sprue and Celiac Disease, *Arch Int Med* 58 1, 1936
- 12 Thaysen, T E H Non tropical Sprue, A Study in Idiopathic Steatorrhea, London, 1932, Humphrey Milford

---

## TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATES OF HEALTHY ADOLESCENTS FIFTEEN TO EIGHTEEN YEARS OF AGE\*

EDWIN C OSGOOD, M D, RUSSELL L BAKER M D INEZ E BROWNLEE, B A,  
MABLE W OSGOOD, B A, DOROTHY M ELLIS B A, AND  
WILLIAM COHEN, M D PORTLAND ORE

---

IT IS the purpose of this article to supply more accurate data than have heretofore been available for total, differential and absolute leucocyte counts, and for sedimentation rates in healthy persons fifteen to eighteen years of age. It is one of a series of papers on hematologic standards for healthy persons from birth through adult life. The data for other age groups will be reported elsewhere.<sup>1</sup>

Search of the literature failed to reveal a single study by modern methods of the total, differential, and absolute leucocyte counts of healthy persons in the age group under consideration, nor were studies found of sedimentation rates by the method here used and recommended.

**Subjects**—All the persons studied lived in or near Portland, Ore., at an elevation of less than 500 feet. All were white and native born in most instances they were children of native born parents. They were from the public high schools of the city of Portland. Adolescents fifteen to eighteen years of age were grouped together because the scatter diagrams<sup>1a</sup> of these values showed no significant differences for the two sexes and no marked variation with age in this group. Children under eight years of age<sup>1b</sup> have higher leucocyte counts, children eight to fourteen years of age<sup>1c</sup> have similar leucocyte counts, but different differential counts, and adults<sup>1d</sup> have lower leucocyte counts and a different differential formula.

### METHODS

Since the methods used have already been described in detail,<sup>2</sup> they will be commented on only briefly here. Venous blood mixed with 2 mg of dry potassium oxalate per cubic centimeter was used for all determinations and is recommended for use because it is more convenient and accurate than use of blood

\*From the Department of Medicine, University of Oregon Medical School, Portland, Ore.  
Received for publication October 17 1938



TABLE I  
TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATE IN FIFTEEN-YEAR-OLD ADOLESCENTS

LEUCO- CYTES* PER C.M.M.	NEUTROPHILE LOBOCYTES*		NEUTROPHILE RHABDOCYTES*		EOSINOPHILE LOBOCYTES		BASOPHILE LOBOCYTES		LYMPHOCYTES		MONOCYTES		DISINTEGRATED CELLS		SEDIMENTATION RATE	
	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*		
Boys																
4.8	50	2.4	1	0.05	1	0.05	0	0.00	42	2.0	2	0.10	4	0.19	1.0	
5.1	42	2.1	0	0.00	1	0.05	1	0.05	48	2.4	4	0.20	4	0.20	0.0	
6.6	38	2.5	2	0.13	3	0.20	1	0.07	48	3.2	4	0.26	4	0.26	1.0	
6.8	59	4.0	1	0.07	1	0.07	2	0.14	30	2.0	4	0.27	3	0.20	1.5	
8.2	34	2.8	1	0.08	4	0.33	0	0.00	53	4.3	4	0.33	4	0.33	1.0	
8.3	46	3.8	0	0.00	2	0.17	1	0.08	42	3.5	4	0.33	4	0.33	1.0	
9.3	27	2.5	0	0.00	0	0.00	0	0.00	58	5.4	5	0.46	10	0.93	6.5	
9.4	52	4.9	2	0.19	1	0.09	2	0.19	40	3.8	1	0.09	2	0.19	0.5	
10.5	56	5.9	1	0.11	2	0.21	1	0.11	34	3.6	4	0.42	2	0.21	2.0	
12.2	57	7.0	2	0.24	4	0.49	0	0.00	26	3.2	10	1.22	1	0.12	8.0	
8.12	46.1	3.79	1.0	0.09	1.9	0.17	0.8	0.06	42.1	3.34	4.2	0.37	3.8	0.30	1.6	
12.2	59	7.0	2	0.24	4	0.49	2	0.19	58	5.4	10	1.22	10	0.93	8.0	
4.8	27	2.1	0	0.00	0	0.00	0	0.00	26	2.0	1	0.09	1	0.12	0.0	
Girls																
4.7	50	2.4	2	0.09	7	0.33	0	0.00	35	1.6	4	0.19	2	0.09	7.0	
5.2	50	2.6	0	0.00	1	0.05	2	0.10	43	2.2	2	0.10	2	0.10	1.5	
6.0	50	3.0	1	0.06	3	0.13	2	0.12	34	2.0	5	0.30	5	0.30	6.0	
6.1	20	1.2	1	0.06	7	0.43	1	0.06	62	3.8	6	0.37	3	0.18	0.5	
6.7	56	3.8	2	0.13	4	0.27	0	0.00	30	2.0	6	0.40	2	0.13	4.5	
6.8	56	3.8	2	0.14	2	0.14	2	0.14	34	2.3	2	0.14	2	0.14	1.0	
7.0	44	3.1	0	0.00	0	0.00	0	0.00	50	3.5	2	0.14	4	0.28	3.0	
7.2	59	4.2	0	0.00	1	0.07	1	0.07	32	2.3	3	0.22	4	0.29	2.0	
8.0	46	3.7	0	0.00	2	0.16	0	0.00	44	3.5	4	0.32	4	0.32	Q.N.S.	
8.0	50	4.0	1	0.08	0	0.00	0	0.00	44	3.5	2	0.16	3	0.24	0.5	
8.3	50	4.2	1	0.08	0	0.00	0	0.00	38	3.2	8	0.65	2	0.17	2.0	
9.5	40	3.8	2	0.19	1	0.10	0	0.00	53	5.0	2	0.19	2	0.19	1.5	
10.0	45	4.5	1	0.10	5	0.50	0	0.00	44	4.4	3	0.30	2	0.20	6.0	
11.6	62	7.2	2	0.23	1	0.12	1	0.12	26	3.0	6	0.70	2	0.23	2.5	
7.51	48.4	3.68	1.1	0.08	2.4	0.17	0.6	0.04	40.6	3.02	3.9	0.30	2.8	0.20	1.9	
11.6	62	7.2	2	0.23	7	0.50	2	0.14	62	5.0	8	0.70	5	0.32	7.0	
4.7	20	1.2	0	0.00	0	0.00	0	0.00	26	1.6	2	0.10	2	0.09	0.5	
Boys and Girls																
Boys	5.12	46.1	3.79	1.0	0.09	1.9	0.17	0.8	0.06	42.1	3.34	4.2	0.37	3.8	0.30	1.6
Girls	7.51	48.4	3.68	1.1	0.08	2.4	0.17	0.6	0.04	40.6	3.02	3.9	0.30	2.8	0.20	1.9
Av.	7.77	47.5	3.72	1.0	0.08	2.2	0.17	0.7	0.05	41.3	3.15	4.0	0.33	3.9	0.24	1.8
Max.	12.2	62	7.2	2	0.24	7	0.50	2	0.19	58	5.4	10	1.22	10	0.93	8.0
Min.	4.7	20	1.2	0	0.00	0	0.00	0	0.00	26	1.6	2	0.09	2	0.09	0.5

TOTAL DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATE IN SIXTEEN YEAR OLD ADOLSCENTS

IFUCO CNTS*	NEUTROPHIL ILOCYTES	LYMPHOCYTES	EOSINOPHIL ILOCYTES	BASOPHIL ILOCYTES	LYMPHOCYTES	MONOCYTES	DISINTEGRATED CPIS		SEDIMENTATION RATE	
	%	%	%	%	%	%	PER CMM*	PER CMM*	15 MIN	45 MIN
49	54	26	0	0	0	0	0.00	0.00	0.5	1.5
54	40	22	0	0	0	0	0.32	0.32	1.0	5.0
60	36	22	4	1	0	0	0.06	0.06	2.0	15.0
67	30	19	1	0	0	0	0.06	0.06	2.0	6.0
67	30	20	2	1	0	0	0.13	0.13	2.0	2.0
71	31	22	1	2	0	0	0.07	0.07	1.0	2.0
84	35	29	0	0	0	0	0.14	0.14	0.5	1.5
86	46	40	0	0	0	0	0.50	0.50	0.5	1.5
86	48	41	0	0	0	0	0.09	0.09	1.0	5.0
90	63	58	1	0	0	0	0.17	0.17	0.5	1.5
158	41	65	1	0	0	0	0.09	0.09	0.5	1.5
750	41	33	2	0	0	0	0.16	0.16	0.5	1.5
158	65	65	2	0	0	0	0.32	0.32	0.5	1.5
49	80	19	0	0	0	0	0.00	0.00	0.5	1.5
60	53	33	1	0	0	0	0.06	0.06	0.5	1.5
60	42	25	2	1	0	0	0.12	0.12	0.5	1.5
69	19	13	0	1	0	0	0.07	0.07	0.5	1.5
74	32	24	1	0	0	0	0.07	0.07	0.5	1.5
75	30	27	0	0	0	0	0.07	0.07	0.5	1.5
84	38	32	1	0	0	0	0.08	0.08	0.5	1.5
87	50	44	2	0	0	0	0.17	0.17	0.5	1.5
91	65	59	0	0	0	0	0.09	0.09	0.5	1.5
91	43	46	0	0	0	0	0.00	0.00	0.5	1.5
94	37	35	1	0	0	0	0.09	0.09	0.5	1.5
104	72	54	5	0	0	0	0.32	0.32	0.5	1.5
106	17	71	3	1	0	0	0.11	0.11	0.5	1.5
82	42	38	1	0	0	0	0.12	0.12	0.5	1.5
106	67	71	3	1	0	0	0.52	0.52	0.5	1.5
60	19	1	0	0	0	0	0.00	0.00	0.5	1.5
789	41	33	10	0	0	0	0.08	0.08	0.5	1.5
832	42	38	14	0	0	0	0.12	0.12	0.5	1.5
811	47	36	12	0	0	0	0.11	0.11	0.5	1.5
178	67	71	5	0	0	0	0.52	0.52	0.5	1.5
19	19	13	0	0	0	0	0.00	0.00	0.5	1.5

\*In thousands

TABLE III  
TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATE IN SEVENTEEN-YEAR-OLD ADOLESCENTS

LEUCOCYTES* PER C.M.M.	NEUTROPHILE LOBOCYTES		NEUTROPHILE RHAOCYTES		EOSINOPHILE LOBOCYTES		BASOPHILE LOBOCYTES		LYMPHOCYTES		MONOCYTES		DISINTEGRATED CELLS		SEDIMENTATION RATE	
	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	15 MIN.	45 MIN.
<i>Boys</i>																
5.0	23	1.2	0	0.00	3	0.15	1	0.05	64	3.2	3	0.15	5	0.25	0.5	2.5
7.6	29	2.2	0	0.00	2	0.15	0	0.00	59	4.5	3	0.23	7	0.53	2.0	10.0
7.7	46	3.5	1	0.08	4	0.31	1	0.08	42	3.2	4	0.31	2	0.15	1.5	5.0
7.9	41	3.2	1	0.08	1	0.08	1	0.08	48	3.8	6	0.47	2	0.16	0.5	1.0
8.1	48	3.9	1	0.08	3	0.24	1	0.08	40	3.2	3	0.24	4	0.32	1.0	5.5
8.5	35	3.0	2	0.17	4	0.34	1	0.08	50	4.2	2	0.17	6	0.51	1.0	2.0
8.7	50	4.4	0	0.00	0	0.00	2	0.17	41	3.6	4	0.35	3	0.26	1.5	8.5
8.7	46	4.0	3	0.26	2	0.17	0	0.00	42	3.7	3	0.26	4	0.35	1.0	4.0
8.9	40	3.6	2	0.18	2	0.18	1	0.09	48	4.3	4	0.36	3	0.27	0.5	1.0
11.4	62	7.1	1	0.11	4	0.46	2	0.23	22	2.5	5	0.57	4	0.46	0.5	7.0
8.25	42.0	3.61	1.1	0.10	2.5	0.21	1.0	0.09	45.6	3.62	3.7	0.31	4.0	0.33	1.0	4.6
11.4	62	7.1	3	0.26	4	0.46	2	0.23	64	4.5	6	0.57	7	0.53	2.0	10.0
5.0	23	1.2	0	0.00	0	0.00	0	0.00	22	2.5	2	0.15	2	0.15	0.5	1.0
<i>Girls</i>																
6.4	41	2.6	0	0.00	2	0.13	0	0.00	49	3.1	3	0.19	5	0.32	1.0	7.0
6.7	30	3.4	0	0.00	2	0.13	1	0.07	40	2.7	4	0.27	3	0.20	0.5	6.0
6.9	38	2.6	0	0.00	0	0.00	0	0.00	51	3.5	5	0.34	6	0.41	1.5	8.0
8.4	52	4.4	0	0.00	2	0.17	1	0.08	42	3.5	1	0.08	2	0.17	1.5	8.5
8.6	54	4.6	1	0.09	0	0.09	0	0.00	39	3.4	2	0.17	3	0.26	2.0	11.0
9.0	55	5.0	1	0.09	2	0.18	1	0.09	32	2.9	8	0.72	1	0.09	1.0	6.0
10.3	54	5.6	0	0.00	1	0.10	1	0.10	41	4.1	2	0.21	2	0.21	0.5	5.0
10.5	42	4.4	1	0.11	1	0.11	0	0.00	46	4.8	5	0.52	5	0.52	2.0	6.0
11.5	38	6.7	2	0.23	0	0.00	0	0.00	34	3.9	5	0.58	1	0.12	1.5	9.0
13.5	64	8.6	0	0.00	2	0.27	1	0.14	23	3.1	6	0.81	4	0.54	2.0	12.0
9.18	50.8	4.79	0.5	0.05	1.3	0.12	0.5	0.05	39.6	3.5	4.1	0.39	3.2	0.28	1.4	7.8
13.5	64	8.6	0	0.23	2	0.27	1	0.14	51	4.8	8	0.81	6	0.54	2.0	12.0
6.4	38	2.6	0	0.00	0	0.00	0	0.00	23	2.7	1	0.08	1	0.09	0.5	5.0
<i>Boys and Girls</i>																
8.25	12.0	3.61	1.1	0.10	2.5	0.21	1.0	0.09	45.6	3.62	3.7	0.31	4.0	0.33	1.0	4.6
9.18	50.8	4.79	0.5	0.05	1.3	0.12	0.5	0.05	39.6	3.50	4.1	0.39	3.2	0.28	1.4	7.8
8.72	46.1	1.30	0.8	0.72	1.9	0.46	0.8	0.07	42.6	3.50	3.9	0.35	3.6	0.30	1.2	6.2
13.5	64	8.6	3	0.26	4	0.46	2	0.23	64	4.8	8	0.81	7	0.51	2.0	12.0
5.0	23	1.2	0	0.00	0	0.00	0	0.00	22	2.5	1	0.08	1	0.09	0.5	1.0

\*In thousands.

TABLE IV  
TOTAL, DIFFERENTIAL AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATE IN EIGHTEEN YEAR OLD CHILDREN

IFUCO CYTES* PER CMM	NEUTROPHIL LOBOCYTES		NEUTROPHIL RHABDOCYTES		EOSINOPHIL LOBOCYTES		BASOPHIL LOBOCYTES		LYMPHOCYTES		MONOCYTES		DISINTEGRATED CELLS		SEDIMENTATION RATE	
	%	IER CMM*	%	PER CMM*	%	PER CMM*	%	PER CMM*	%	IER CMM*	%	PER CMM*	%	PER CMM*	15 MIN	45 MIN
<i>Boys</i>																
68	37	25	2	0.14	9	0.61	0	0.00	42	2.9	2	0.14	8	0.54	25	60
74	54	40	2	0.15	0	0.00	1	0.07	73	2.4	6	0.44	4	0.30	05	15
77	41	32	0	0.00	8	0.62	2	0.15	42	2.4	4	0.31	3	0.23	05	15
82	36	30	0	0.00	1	0.08	0	0.00	56	4.6	3	0.25	4	0.33	15	45
82	37	30	0	0.00	1	0.08	0	0.00	52	4.3	6	0.49	4	0.33	05	25
84	53	45	2	0.17	1	0.08	1	0.09	37	3.1	2	0.17	4	0.34	30	110
86	48	41	0	0.00	0	0.00	1	0.09	41	3.5	6	0.52	4	0.34	10	32
88	46	40	1	0.09	1	0.09	1	0.09	44	3.9	4	0.35	2	0.18	20	100
110	38	42	0	0.00	2	0.22	1	0.11	48	5.3	6	0.66	5	0.55	15	95
113	48	54	2	0.23	4	0.45	0	0.00	38	4.3	4	0.45	3	0.34	40	100
861	43.8	379	0.9	0.08	27	0.32	0.7	0.06	43.3	7.5	4.3	0.38	4.1	0.45	17	37
113	54	54	2	0.23	0	0.02	2	0.15	56	5.3	6	0.66	8	0.53	40	100
68	36	25	0	0.00	0	0.00	0	0.00	33	2.4	2	0.14	2	0.18	05	15
<i>Girls</i>																
40	13	13	0	0.00	0	0.20	0	0.00	61	2.4	1	0.04	0	0.00	10	60
62	58	36	0	0.00	3	0.19	1	0.06	48	2.0	6	0.37	0	0.00	10	70
66	40	26	0	0.00	6	0.40	2	0.13	30	2	4	0.26	0	0.00	10	100
67	61	41	0	0.00	0	0.00	1	0.07	45	1.9	9	0.60	0	0.00	30	220
74	43	32	5	0.27	0	0.00	0	0.00	45	3.3	7	0.52	0	0.00	20	260
74	15	48	0	0.00	0	0.00	1	0.07	44	4	2	0.15	0	0.00	20	110
77	40	31	0	0.00	2	0.15	0	0.00	44	4	6	0.46	8	0.62	50	230
96	19	16	0	0.00	0	0.00	0	0.00	5	7	3	0.29	0	0.00	40	200
112	58	65	1	0.11	7	0.79	0	0.00	29	3.2	5	0.56	0	0.00	60	320
120	59	71	1	0.12	0	0.00	0	0.00	3	4.0	7	0.54	0	0.00	25	190
788	42.6	429	0.7	0.06	2.3	0.17	0.5	0.03	38.1	2.85	5.0	0.41	0.8	0.06	28	178
120	19	71	5	0.37	7	0.79	2	0.13	61	4.0	9	0.84	8	0.62	60	320
40	13	13	0	0.00	0	0.00	0	0.00	28	1.9	1	0.04	0	0.00	10	60
<i>Boys and Girls</i>																
464	4.8	179	0.9	0.08	27	0.22	0.7	0.06	43.3	7.5	4.3	0.38	4.1	0.35	17	37
788	52.6	429	0.7	0.06	27	0.17	0.5	0.03	38.1	2.85	5.0	0.41	0.8	0.06	28	178
826	18.2	404	0.8	0.07	2.5	0.20	0.6	0.03	40.7	0	4.6	0.39	2.4	0.20	22	117
120	19	71	5	0.37	0	0.79	2	0.15	61	5.3	9	0.84	8	0.62	60	320
10	33	13	0	0.00	0	0.00	0	0.00	28	1.9	1	0.04	0	0.00	05	15

\*in thousands

TABLE V

SUMMARY OF TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATIOS IN ADOLESCENTS FIFTEEN TO EIGHTEEN YEARS OF AGE

AGE	NO. OF CASES	LEUCOCYTES*		NEUTROPHILIC LOBOCYTES		EOSINOPHILIC LOBOCYTES		BASOPHILIC LOBOCYTES		LYMPHOCYTES		MONOCYTES		DISINTEGRATED CELLS		SEDIMENTATION RATE		
		PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	%	PER C.M.M.*	15 MIN. 45 MIN.	
Boys																		
15	10	8.12	46.1	3.79	1.0	0.09	1.9	0.17	0.8	0.06	42.1	3.34	4.2	0.37	3.8	0.30	1.6	8.0
16	11	7.89	41.4	3.31	1.0	0.08	2.3	0.17	0.8	0.06	45.2	3.49	4.7	0.40	4.4	0.36	1.6	7.6
17	10	8.25	42.0	3.61	1.1	0.10	2.5	0.21	1.0	0.09	45.6	3.62	3.7	0.31	4.0	0.33	1.0	4.6
18	10	8.04	43.8	3.79	0.9	0.08	2.7	0.22	0.7	0.06	43.3	3.73	4.3	0.38	4.1	0.35	1.7	5.7
41		8.22	43.3	3.62	1.0	0.09	2.3	0.19	0.8	0.07	44.1	3.55	4.2	0.37	4.1	0.33	1.5	6.5
		15.8	65.0	7.1	3.0	0.32	9.0	0.62	2.0	0.23	61.0	6.8	11.0	1.42	10.0	0.93	8.0	43.0
		4.8	23.0	1.2	0.0	0.00	0.0	0.00	0.0	0.00	21.0	1.8	1.0	0.09	1.0	0.05	0.0	1.0
Girls																		
15	14	7.51	48.4	3.68	1.1	0.08	2.4	0.17	0.6	0.04	40.6	3.02	3.9	0.30	2.8	0.20	1.9'	11.4'
16	12	8.32	45.2	3.86	1.4	0.12	1.3	0.10	0.6	0.05	43.2	3.51	4.6	0.38	3.8	0.30	1.8	9.8
17	10	9.18	50.8	4.79	0.5	0.05	1.3	0.12	0.5	0.05	39.6	3.50	4.1	0.39	3.2	0.28	1.4	7.8
18	10	7.88	52.6	4.29	0.7	0.06	2.3	0.17	0.5	0.03	38.1	2.85	5.0	0.41	0.8	0.06	2.8	17.8
46		8.16	49.0	4.10	1.0	0.08	1.9	0.14	0.6	0.04	40.5	3.22	4.4	0.36	2.7	0.22	1.9''	11.3''
		13.5	69.0	8.6	5.0	0.52	7.0	0.79	2.0	0.14	72.0	5.0	9.0	0.84	8.0	0.62	7.0	36.5
		4.0	19.0	1.2	0.0	0.00	0.0	0.00	0.0	0.00	22.0	1.6	1.0	0.04	0.0	0.00	0.5	2.0
Boys and Girls																		
13 cases: " 45 cases.																		
41		8.22	43.3	3.62	1.0	0.09	2.3	0.19	0.8	0.07	44.1	3.55	4.2	0.37	4.1	0.33	1.5	6.5
46		8.16	49.0	4.10	1.0	0.08	1.9	0.14	0.6	0.04	40.5	3.22	4.4	0.36	2.7	0.22	1.9'	11.3'
87		8.19	46.3	3.87	1.0	0.08	2.1	0.16	0.7	0.05	42.2	3.37	4.3	0.36	3.4	0.27	1.7''	9.1''
		15.8	69.0	8.6	5.0	0.52	9.0	0.79	2.0	0.23	72.0	6.8	11.0	1.12	10.0	0.93	8.0	43.0
		4.0	19.0	1.2	0.0	0.00	0.0	0.00	0.0	0.00	21.0	1.6	1.0	0.04	0.0	0.00	0.0	1.0

\* 13 cases; " 45 cases.

\*In thousands. " 15 cases; " 86 cases.

TABLE VI

STATISTICAL AND SMOOTHED MEANS AND RANGES FOR TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATES IN ADOLESCENTS FIFTEEN TO EIGHTEEN YEARS OF AGE

		STATISTICAL MEAN	PROBABLE ERROR DISTRIBUTION	SMOOTHED MEAN	95% RANGE
Leucocytes*	per c mm	5,370 ± 91	1541 ± 47	8400	4,000 13,000
Neutrophile lobocytes†	per cent	47.4 ± 0.7	7.7 ± 0.3	48	25 70
	per c mm	3,937 ± 94	Sl ew	4000‡	1500 7,500‡
Neutrophile rhabdocytes	per cent			1\$	0 5\$
	per c mm			80	0 200\$
Eosinophile lobocytes	per cent			2\$	0 6\$
	per c mm			100\$	0 400\$
Basophile lobocytes	per cent			0.5	0 2
	per c mm			50	0 200
Lymphocytes‡	per cent	41.8 ± 0.6	10.3 ± 0.4	42	22 62
	per c mm	3,304 ± 57	Sl ew	3200	1500 5,000
Monocytes	per cent			4\$	0 9\$
				300	0 800
Disintegrated cells	per cent			3\$	0 7
	per c mm			250\$	0 600\$
Sedimentation rate	15 min		Skew	20	0 550
	45 min		Sl ew	100	10 300

\*Applies to persons 8 to 18 years of age

†Applies to persons 15 to 19 years of age

‡Applies to persons 4 to 7 and 15 years of age and over

\$Applies to persons 15 years of age and over

||Applies to persons 4 years of age and over

from the finger or ear. No difficulty was encountered in obtaining blood from the vein. The blood was taken at any time of day as in ordinary office practice.

The leucocyte counts were made with apparatus certified by the Bureau of Standards, counting an area of 0.4 cmm on each of two dilutions and using all the precautions outlined by Osgood.<sup>2</sup> The differential cell counts were made on Wright's stained smears on glass slides. Buffer phosphate was used as a diluent. The details of smearing and staining are described elsewhere.<sup>3</sup> In making the differential counts, 200 or more cells were classified according to the criteria described and illustrated in *An Atlas of Hematology*,<sup>3</sup> taking care to study only such portions of the smears as were thin enough so that the akaryocytes (erythrocytes) were not touching each other. The absolute leucocyte counts were calculated by multiplying the total leucocyte count by the percentage of the particular cell type. Plum<sup>4</sup> has given data from which the probable error of any of these determinations may be calculated.

The sedimentation rates were determined on the same samples of oxalated blood, using a Westergren pipette, and taking readings at fifteen and forty five minutes. Experience with over 80,000 determinations by this method has shown it to have the advantages of the graphic methods and to be simpler and quicker.

## RESULTS

Tables I to IV summarize the individual results for the total, differential, and absolute leucocyte counts and sedimentation rates for each age and sex group. On examination of these tables and from the scatter diagrams,<sup>1d</sup> it is evident that there is no significant difference with age or sex within this group, so that it is justifiable to summarize the results for the entire group as has been

done in Table V. This table gives the averages and extreme ranges. For clinical purposes, however, the most useful range of normal is not the extreme range, but that range which will include about plus or minus three probable errors, or 95 per cent of healthy individuals. In other words, if a result in a person of this age group falls outside of the range, there is less than one chance in twenty that it is normal for that person. These 95 per cent ranges are summarized in Table VI.

The sedimentation rates form a skew curve, with the greater number of determinations falling in the lower rates. It is probable that the rate of 15 mm. in forty-five minutes, which includes 80 per cent of the results, represents the strict upper limits of normal, and that the higher rates are due to mild chronic infection in the tonsils, teeth, or sinuses not detectable in the routine physical examination.

#### SUMMARY

There are no significant age or sex differences in the total, differential, or absolute leucocyte counts or in the sedimentation rates in persons fifteen to eighteen years of age. Table VI gives the data which should be most useful in interpretation of each of these determinations in persons of this age group. This is apparently the first study of normal values for these determinations in adolescents fifteen to eighteen years of age.

#### REFERENCES

- 1a. Osgood, E. E.: Hemoglobin, Color Index, Saturation Index, and Volume Index Standards, *Arch. Int. Med.* 37: 685, 1926.
- b. —, and Haskins, H. D.: The Relation Between Cell Count, Cell Volume, and Hemoglobin Content of the Venous Blood of Normal Young Women, *Arch. Int. Med.* 39: 643, 1927.
- c. —, and Baker, R. L.: Erythrocyte, Hemoglobin, Cell Volume and Color, Volume and Saturation Index Standards for Normal Children of School Age, *Ann. J. Dis. Child.* 50: 343, 1935.
- d. Osgood, E. E.: Normal Hematologic Standards, *Arch. Int. Med.* 56: 849, 1935.
- e. —, and others: Total, Differential and Absolute Leukocyte Counts and Sedimentation Rates for Healthy Children Four to Seven Years of Age. To be published.
- f. Idem: Total, Differential and Absolute Leukocyte Counts and Sedimentation Rates for Healthy Children Eight to Fourteen Years of Age. To be published.
- g. Idem: Total, Differential and Absolute Leukocyte Counts and Sedimentation Rates for Healthy Adults Nineteen Years of Age and Over. To be published.
- h. Chuihard, E. G., Osgood, E. E., and Ellis, Dorothy M.: Total, Differential and Absolute Leukocyte Counts and Sedimentation Rates for Healthy Newborn Infants. To be published.
- i. Idem: Erythrocyte Counts, Hemoglobin, Cell Volume, and Color, Volume and Saturation Index Standards for Healthy Newborn Infants. To be published.
- j. Osgood, E. E., and others: Erythrocyte Counts, Hemoglobin, Cell Volume, and Color Volume and Saturation Index Standards for Healthy Adolescent Males Fifteen Years of Age and Over. To be published.
- k. Idem: Erythrocyte Counts, Hemoglobin, Cell Volume, and Color, Volume and Saturation Index Standards for Healthy Females Fifteen Years of Age and Over. To be published.
- l. Idem: Reticulocyte Counts in Healthy Newborn Infants. To be published.
- m. Idem: Icterus Index Determinations in Healthy Newborn Infants. To be published.
- n. Idem: Reticulocyte Counts in Healthy Adolescent Boys and Girls. To be published.
2. Idem: A Textbook of Laboratory Diagnosis, ed. 2, Philadelphia, 1935, P. Blakiston's Son & Co., pp. 409-415 and 430-432.
3. Osgood, E. E., and Ashworth, Clarice M.: An Atlas of Hematology, San Francisco, 1937, J. W. Stacey, Inc., p. 255.
4. Plum, P.: Accuracy of Hematological Counting Methods, *Acta. med. Scandinav.* 90: 342, 1936.

# BIOCHEMICAL OBSERVATIONS IN HYPOGLYCEMIA INDUCED BY INSULIN\*

## IV SOME EVIDENCES OF BLOOD CONCENTRATION IN HYPOGLYCEMIA

EDWARD F. ROSENBERG, M.D. ROCHESTER MINN.

IN A REPORT of the behavior of the blood gases during hypoglycemia<sup>6</sup> I pointed out that the hypoglycemic state is frequently associated with the development of an increase in oxygen carrying capacity of venous blood. I stated that this increase is probably due to hemoconcentration and that studies of the hemoglobin should reveal an increasing value as hypoglycemia appeared.

### CONCENTRATION OF HEMOGLOBIN

*Method*—Five patients were studied in the following manner. At 7:00 A.M. blood was drawn from an elbow vein and measured quantities of exactly 0.1 cc (Kahn pipette) were immediately transferred to 20 cc of 0.1 per cent solution of sodium carbonate in bottles impervious to actinic rays. A "coma dose" of insulin was injected into each patient after the withdrawal of the first sample of venous blood. Thereafter specimens of venous blood were collected hourly using the same procedure as above. The hypoglycemic state was interrupted at 11 A.M. (at which time all 5 patients had been in hypoglycemic coma for approximately one and a half hours) by the administration of 150 gm of sucrose in water, through a nasal tube. The noon meal was served and a final blood specimen was collected at 2 P.M. The samples for hemoglobin determination were then sent to the Section on Clinical Pathology of the Mayo Clinic where they were examined by the photoelectric method<sup>8</sup> (Table I).

Four of the 5 cases studied showed a definite rise in the value of hemoglobin during hypoglycemia (Fig. 1). The average value for hemoglobin begins at 12.9 gm per 100 cc of blood at 7 A.M. (86 per cent on a basis of 15 gm for 100 per cent) and reaches a high point of 13.8 gm at 11 A.M. (92 per cent). Three hours after the termination of the hypoglycemia the average value had fallen to 13.2 gm, or 88 per cent. The total rise was 7.0 per cent over the initial value. This is in agreement with the order of increase in value of oxygen capacity noted in my data on the blood gases in hypoglycemia.

*Comment*—Diabkin and Ravdin have published charts of the changes in hemoglobin values in dogs during hypoglycemia. These charts indicate that the hemoglobin rises 20 to 40 per cent during hypoglycemia. However these

\*From the Mayo Foundation, Rochester, Minn.

Abridgment of thesis submitted to the Graduate School of The University of Minnesota in partial fulfillment of the requirements for the degree of M.S. in Medicine.

Received for publication October 20, 1933.



workers used enormous doses of insulin (20 units per kg. of body weight, equivalent to 1,400 units in a man weighing 70 kg.) and these results should strictly not be compared with our own observations, which were made using doses of the order of 1 to 2 units per kg. of body weight.

Wiechmann and Koeh studied the variations in hemoglobin content of blood of human beings in relation to the intra-ocular tension in hypoglycemia. After injection of a "toxic" dose of insulin (150 units in one instance and 100 units in the second) into fasting patients, there appeared a considerable rise of the hemoglobin which fell after the taking of food. Their charts showed a rise from approximately 14 gm. to 18 gm. in one instance. This rise occurred over a period of five hours. A second chart pictures a rise from approximately 15.5 gm. to 18 gm. They believed that this change resulted from the perspiration which accompanied the hypoglycemia. This observation was restated in a subsequent paper by Wiechmann and Liang.

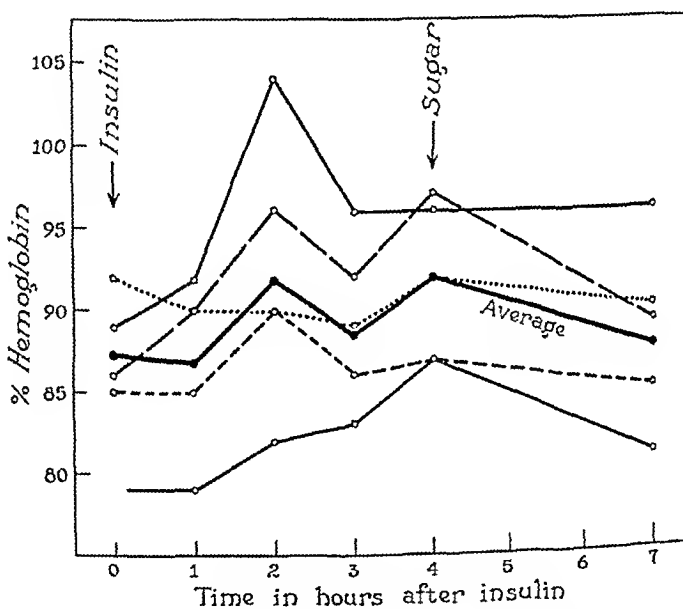


Fig. 1.—Level of hemoglobin of venous blood in relation to hypoglycemia induced by insulin.

#### HEMATOCRIT AND THE RED CORPUSCLE COUNT IN HYPOGLYCEMIA

In the two studies represented in Table II, the hematocrit reading is seen to increase as hypoglycemia develops. The value of hematocrit tends to remain elevated above the original morning level late in the afternoon following a morning treatment. This tendency to remain elevated was also noted in studies of the oxygen capacity and hemoglobin in relation to hypoglycemia. It suggests that the hypoglycemic state is associated with changes in the physiologic status of the organism which persist for hours after the blood sugar has been elevated to normal levels by means of the administration of sugar.

A few observations of the red blood corpuscle count were made in relation to hypoglycemia (Table III). With these counts simultaneous hematocrit and

TABLE I

RELATIONSHIP OF THE VALUE OF HEMOGLOBIN TO THE HYPOLYCEMIC STATE

TIME	BLOOD SUGAR*	HEMOGLOBIN	PER CENT HEMOGLOBIN
	Mg per 100 cc of blood	Gm per 100 cc of blood	(15 gm = 100%)
Patient 1 Coma dose of insulin 100 units at 7 A M			
7 A M	107.5	12.7	85
8 A M		12.7	85
9 A M	21.0	13.5	90
10 A M		12.9	86
11 A M	20.0	13.1	87
2 P M	131.6	12.7	85
Patient 2 Coma dose of insulin 120 units at 7 A M			
7 A M	133.3	13.8	92
8 A M		13.5	90
9 A M	20.0	13.5	90
10 A M		13.3	89
11 A M	20.0	13.8	92
2 P M	104.7	13.3	89
Patient 3 Coma dose of insulin 120 units at 7 A M			
7 A M	131.6	13.3	89
8 A M		13.8	92
9 A M	24.0	15.5	103
10 A M		14.4	96
11 A M	20.1	14.4	96
2 P M	111.1	14.4	96
Patient 4 Coma dose of insulin 120 units at 7 A M			
7 A M	104.2	11.6	77
8 A M		11.7	78
9 A M	20.0	12.2	82
10 A M		12.5	83
11 A M	23.0	13.1	87
2 P M	129.0	12.1	81
Patient 5 Coma dose of insulin 120 units at 7 A M			
7 A M	86.0	12.0	86
8 A M		13.5	90
9 A M	20.0	14.4	96
10 A M		13.8	92
11 A M	20.0	14.6	97
2 P M	100.0	13.3	89

\*Micromethod of Folin

blood sugar studies were made. In these studies the rise in hematocrit with the appearance of hypoglycemia was repeated, and there was an increase in the red blood corpuscle count. The extent of change in these counts, although strictly not large enough to be of statistical significance, is nevertheless probably accurate in these instances.

As a control of the above observations the following study was made (Table IV) without administration of insulin. Thus, without insulin and without hypoglycemia there is no significant change in the value of hematocrit or of the red blood corpuscle count.

*Comment*—The finding of an increase in oxygen capacity and an increase in the hemoglobin concentration of the blood during hypoglycemia led to a study of the behavior of hematocrit and the red blood count in relation to hypoglycemia.

TABLE II  
RELATIONSHIP OF HEMATOCRIT VALUE TO HYPOLYCEMIA

TIME	BLOOD SUGAR*	HEMATOCRIT READING†	ERYTHROCYTES
	Mg. per 100 c.c. of blood		Vols. per cent
April 4, 1937. Patient 6. 90 units of insulin at 7 A.M.			
7 A.M.	86.9	2.38	47.6
8 A.M.	80.2	2.40	48
9 A.M.	67.8	2.55	51
10 A.M.	33.3	2.6	52
11 A.M.	29.4	2.6	52
11 A.M.	150 gm. sucrose by nasal tube		
1 P.M.	162	2.6	52
4 P.M.	Not done	2.55	51
Patient 7. 160 units of insulin at 7 A.M.			
7 A.M.	120.0	2.3	46
8 A.M.	81.6	2.32	46.6
9 A.M.	40.3	2.35	47
10 A.M.	29.3	2.4	48
11 A.M.	28.8	2.65	53
11 A.M.	150 gm. sucrose by nasal tube		
1 P.M.	133.3	2.65	53
4 P.M.	Not done	2.55	51

\*Micromethod of Folin.

†On basis of 5 c.c. of whole blood.

TABLE III  
RELATIONSHIP OF HEMATOCRIT VALUE AND ERYTHROCYTE COUNT TO HYPOLYCEMIA

TIME	BLOOD SUGAR*	HEMATOCRIT READING†	ERYTHROCYTES	ERYTHROCYTES
	Mg. per 100 c.c. of blood		Vols. per cent	Millions
April 12, 1937. Patient 6. 90 units of insulin at 7:45 A.M.				
7:45 A.M.	88.1	2.2	44	3.71
9:45 A.M.	54.8	2.5	50	3.85
11:45 A.M.	25.0	2.65	53	3.87
11:45 A.M.	150 gm. sucrose by nasal tube			
1:45 P.M.	121.2	2.55	51	3.97
3:45 P.M.	Not done	2.50	50	3.78
April 16, 1937. Patient 8. 100 units of insulin at 7 A.M.				
7:00 A.M.	95.2	2.55	51	4.52
9:00 A.M.	55.5	2.70	54	4.84
11:00 A.M.	23.7	2.95	55	4.90
1:00 P.M.	129.0	2.75	55	4.62
4:00 P.M.	Not done	2.50	50	4.51

\*Micromethod of Folin.

†On basis of 5 c.c. of whole blood.

Klein and Kment have observed that after administration of insulin, if hypoglycemic shock is permitted to develop, evidence of blood concentration appears. They found an increased red blood count, a rise in hematocrit, and an increase in serum albumin and dry substance of the blood in hypoglycemia. They stated that these findings do not appear if the blood sugar does not fall to hypoglycemic levels. Examination of their figures, however, gives one the impression that these conclusions were based on very slight changes. Only in one case did the rise in red blood count amount to 600,000, and even

TABLE IV  
CONTROL OBSERVATION

TIME	BLOOD SUGAR*	HEMATOCRIT READING†	ERYTHROCYTES Fols per cent	EPYTHROCYTES Millions
	Mg per 100 cc of blood			
April 16, 1937 Patient 6 Fasting—No insulin				
7 00 A M	108.1	24	48	3.51
9 00 A M	101.5	25	50	3.61
11 00 A M	95.2	24.5	40	3.46
1 00 P M	114.0	24	48	3.57
4 00 P M	Not done	21	48	3.53

\*Micromethod of Folin

†On basis of 5 cc of whole blood

TABLE V  
RELATIONSHIP OF PLASMA CHLORIDES TO HYPOLYCEMIA

TIME	BLOOD SUGAR*	PLASMA CHLORIDES
	Mg per 100 cc of blood	
May 26, 1937 Patient 1 110 units of insulin at 7 00 A M		
7 00 A M	83.5	578
9 00 A M	71.2	584
11 00 A M	20.0	589
Patient 3 130 units of insulin at 7 00 A M		
7 00 A M	80.0	581
9 00 A M	33.3	591
11 00 A M	30.0	600
May 27, 1937 Patient 2 110 units of insulin at 8 00 A M		
7 00 A M	111.1	595
9 00 A M	25.0	601
11 00 A M	25.0	610
Patient 1 110 units of insulin at 7 00 A M		
7 00 A M	108.7	596
9 00 A M	27.5	603
11 00 A M	23.0	609
September 7, 1937 Patient 9 60 units of insulin at 6 30 A M		
6 30 A M	87.5	594
8 30 A M	29.7	637
10 30 A M	20.0	618

\*Micromethod of Folin

this amount is not significant in a single reading. The hematocrit changes were somewhat more consistent but were nevertheless mostly of a small order.

A rise in the red blood count might be expected in hypoglycemia if we accept the theory of increased excretion of adrenalin in hypoglycemic shock. Hoskins and Gunning have noted that injection of epinephrine causes contraction of the spleen, and this would be expected to result in a rise in the red blood count. Izquierdo and Cannon have reported an emotional polycythemia in cats, and Schneider and Havens have stated that polycythemia results from injection of epinephrine in man.

## THE LEVEL OF CHLORIDES IN PLASMA DURING HYPOLYCEMIA

The observation of the occurrence of hemoconcentration in hypoglycemia in man, together with the marked perspiration which is often characteristic of

hypoglycemic shock, suggested a study of the chlorides of the blood in our patients (Table V).

In each instance the studies of Table V have shown a slight rise in plasma chlorides during hypoglycemia. These findings are in accord with other evidence already presented that a slight hemoconcentration accompanies the hypoglycemic state.

#### SUMMARY AND CONCLUSIONS

My studies confirm the impression gained from considerable scattered evidence in the literature suggesting that the hypoglycemic state is associated with definite hemoconcentration. I have found a considerable rise in the values both for hemoglobin and for oxygen-carrying capacity of venous blood, and also distinct rises in the values for hematocrit and red blood corpuscle count. The levels of plasma chlorides in these same patients have regularly risen during hypoglycemia, and studies (made by Dr. Ancel Keyes) on these patients have shown that in the hypoglycemic state the amount of protein per unit volume of blood is increased. All of these findings indicate that water is lost from the blood, whereas erythrocytes, proteins, and some salts are retained.

#### REFERENCES

1. Drabkin, D. L., and Ravdin, I. S.: Mechanism of Convulsions in Insulin Hypoglycemia. Interrelationship of Blood Concentration, Cerebrospinal Pressure and Convulsions, *Am. J. Physiol.* 118: 174, 1937.
2. Hoskins, R. G., and Gunning, R. E. L.: The Effects of Adrenin on the Distribution of the Blood, *Am. J. Physiol.* 43: 298, 1917.
3. Izquierdo, J. J., and Cannon W. B.: Studies on the Conditions of Activity in Endocrine Glands. XXIII. Emotional Polycythemia in Relation to Sympathetic and Medullary-adrenal Action on the Spleen, *Am. J. Physiol.* 84: 545, 1928.
4. Keyes, Ancel: Personal communication.
5. Klein, O., and Kment, M.: Beobachtungen über Insulin-hypoglykämie beim Menschen; Verhalten der Eiweissfraktionen des Blutes, des Bilirubins, der Blutkonzentration und der Blutgerinnung, insbesondere bei Leberkranken, *Ztschr. f. klin. Med.* 107: 475, 1928.
6. Rosenberg, E. F.: Unpublished data.
7. Schneider, E. C., and Havens, L. C.: Changes in the Blood After Muscular Activity and During Training, *Am. J. Physiol.* 36: 239, 1915.
8. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, ed. 8, Philadelphia, 1935, W. B. Saunders Co., pp. 229-230.
9. Wiechmann, E., and Koch, F.: Untersuchungen über den hypoglykämischen Zustand nach Insulininjektion; über die Ursachen der Augendrucksenkung im hypoglykämischen Zustand, *Deutsches. Arch. f. klin. Med.* 160: 361, 1928.
10. Wiechmann, E., and Liang, Seluwen: Der Wasserhaushalt der Diabetiker und seine Beeinflussung durch Insulin, *Deutsches. Arch. f. klin. Med.* 163: 282, 1929.

## THE INTRAMUSCULAR INJECTION OF VITAMIN K\*

GARNETT CHENFY, M.D. SAN FRANCISCO, CALIF.

IT HAS been conclusively established that a deficiency of vitamin K in the diet of newly hatched chicks will lead to an abnormally prolonged blood coagulation time which becomes manifest in the second or third week of life, and which may be so pronounced that fatal hemorrhage will occur (fowl hemophilia). The addition to the diet of vitamin K or substances containing it, will correct the blood coagulation defect within three days time. The basic studies leading to the elucidation of the nature of this vitamin deficiency disease have been carried out by Dam<sup>1, 2</sup> in Copenhagen, Denmark and by Almquist<sup>3, 4</sup> in Berkeley, California. Recently, Almquist<sup>5</sup> has isolated the vitamin and made it available for use in pure form.

Up until the time the present studies were begun vitamin K had only been used orally. The biological assay of its effectiveness has been carried out by Almquist<sup>7</sup> by determining the amount of vitamin in the diet which was necessary to reduce the blood coagulation time to a normal level after three days of feeding. He set the upper limit of the normal coagulation time of shed blood as thirty minutes, which corresponds with our own experience although the coagulation time is usually less than ten minutes. The average time for 25 range birds of different ages was found to be 6.1 minutes, 76 per cent of the birds being under six minutes. The time intervals varied from one to thirty minutes. Most of the control birds raised in the laboratory have a coagulation time of five minutes or less. Almquist found that unrefined preparations of vitamin K were effective at a level as low as 10 mg. per kg. of diet. Schonheyder has developed a more complicated form of biological assay.<sup>8</sup>

The chief aims of the present study were (1) to determine the feasibility of administering vitamin K by intramuscular injections, (2) the effective parenteral dose which might be required, (3) the length of time it would take such a dose to reduce the blood coagulation time to normal, (4) the toxicity of the product used, and (5) the application of the knowledge obtained from injecting vitamin K into birds to the problem of similarly treating human patients. In April, 1938, Dam and Glavind<sup>9</sup> reported very briefly on the successful administration of vitamin K, parenterally, to birds and man, but failed to include any experimental data which would have any bearing on questions 2 to 5. They state that this data will be published at a later date.

\*From the Department of Medicine, Stanford University Medical School, San Francisco.  
Received for publication December 3, 1938.

Our chicks\* were raised in the laboratory in brooders with screened floors. The day after hatching they were started on a basal diet consisting of rice flour, 73 per cent; ether extracted fish meal,† 17.5 per cent; brewer's yeast, 7.5 per cent; cod-liver oil, 1 per cent; and sodium chloride 1 per cent. The diet is deficient in vitamin K, but may not produce a marked hemorrhagic tendency unless the yeast is also extracted with ether. Great care must be exercised in keeping the cages free from droppings, as the feces are commonly ingested and may contain enough of the vitamin to prevent the development of the desired deficiency. With the exception of 3 birds to be noted later, all the chicks used in our experiments were 3 or 4 weeks old, and as the tables show, the great majority showed a prolonged blood coagulation time. In any series of birds studied, the time will vary from normal in an occasional bird to over twenty-four hours. This may in part be due to the individual susceptibility of different birds, but is probably due to the fact that normal coagulation times occur following spontaneous and nonfatal hemorrhage. This probably also explains the frequency of normal coagulation times as the birds get older and the striking cyclic variations observed. Because of this beneficial effect of bleeding on fowl hemophilia, the birds must be studied in groups and not singly, in order to determine the extent of the bleeding tendency. For the blood coagulation tests 5 to 8 drops of freely flowing blood from a wing vein puncture were received into small clean test tubes which had been rinsed with normal salt solution. The tubes were allowed to stand at room temperature, and coagulation was considered complete when no flow of blood occurred on inverting the tubes.

*The Injection of Vitamin K in Chicks.*—Vitamin K is a fat-soluble, heat-stable sterol, and the preparation used had the appearance of a thin yellow oil. It was supplied by Eli Lilly and Company, and was originally assayed in December, 1937, by Dr. H. J. Almquist, as being active at the 40 mg. level per kg. of basal diet. It contained 270 mg. of vitamin K per c.c. of the liquid substance. Just preceding our first injection experiments with vitamin K, we found this preparation gave complete protection when fed at a level of 54 mg. per kg. of diet, and that the coagulation time of the blood was normal as early as the second day. All the injections were given through a hypodermic needle into the breast muscle. Necessary dilutions for injections in birds were made with petroleum ether. No local reactions occurred. Very large doses were rapidly fatal in chicks, and in three instances death occurred following the injections of small doses. Autopsies failed to show the cause of the fatalities, although in some, hemorrhage into the liver suggested the injecting needle had passed through the rib cage into liver tissue.

Table I shows the results of injecting fairly large doses of vitamin K (6.5 mg. and 2.7 mg.) intramuscularly in 25 chicks. The vitamin preparation was autoclaved before injecting it into the first 17 chicks. The age of the birds varied from 17 days to 28 days, and the weight from 75 gm. to 140 gm., the older birds being heavier on the average. The average weight of the chicks at three weeks of age was about 100 gm. Where initial blood coagulation times

\*We are indebted to Poehlmann Hatchery in Petaluma, Calif., for donating all of the chicks used in this work.

†We are indebted to Dr. H. J. Almquist of the University of California at Berkeley, for the supply of extracted fish meal used in the diet.

TABLE I

THE EFFECT OF VITAMIN K ADMINISTERED INTRAMUSCULARLY IN REDUCING THE COAGULATION TIME OF THE BLOOD OF CHICKS WITH FOWL HEMOPHILIA

BIRDS	AGE DAYS	INITIAL COAG ULATION TIME	VITAMIN K AMOUNT	COAGULATION TIMES AFTER INJECTION
				1 HR      6 HR      25 HR
<i>Group I</i>				
VI 82	17		6.5 mg	10
XI 90	17		6.5 mg	7
VI 73	17		6.5 mg	1
VI 106	17		6.5 mg	3
XI 75	17		6.5 mg	4
<i>Group II</i>				
VI 98	20		2.7 mg	5
VI 36	20		2.7 mg	1
XI 91	20		2.7 mg	5
XI 97	20		2.7 mg	2
XI 87	20		2.7 mg	2
VI 89	20		2.7 mg	2
<i>Group III</i>				
VI 35	27	50	2.7 mg	10
XI 12	27	25	2.7 mg	7
VI 86	27	11	2.7 mg	5
XI 101	27		2.7 mg	1
XI 11	27		2.7 mg	6
XI 24	27		2.7 mg	2
VI 106	21	61	2.7 mg	21
VI 107	21	102	2.7 mg	1
VI 108	21	52	2.7 mg	1
VI 109	21	>6 hours	2.7 mg	1
VI 110	21	>6 hours	2.7 mg	16
VI 111	21	>6 hours	2.7 mg	3
XI 112	21	>6 hours	2.7 mg	15
VI 113	21	160	2.7 mg	8

of the groups are not recorded, abnormally prolonged blood coagulation times were established for the whole group by bleeding a number of chicks which did not receive vitamin K. Group I shows a normal blood coagulation in each of 5 birds twenty-four hours after receiving an injection of 6.5 mg of the vitamin, the times varying between one and ten minutes. Similarly in Group II, normal times were found in 6 birds which received 2.7 mg of vitamin K each. Not only was the dose smaller for this group, but the coagulation time was tested at the end of six hours instead of twenty-four hours. In Group III, 14 chicks received the same amount of vitamin K intramuscularly, 2.7 mg, but the blood coagulation times were tested just one hour after the injections. They were well under thirty minutes in all but 2 birds in which the times were thirty-two minutes and twenty-nine minutes and ranged from two to twenty-three minutes, the average for these 12 chicks being 10.6 minutes. In 11 of these 14 birds, initial coagulation times were taken before the injection of vitamin K, and were longer than thirty minutes in all but two instances. The prolonged times varied from fifty minutes to longer than six hours. Table I shows the marked reduction in blood coagulation time which took place in each bird. These experiments in these three groups of chicks strongly suggest that large doses of vitamin K given intramuscularly will reduce an abnormally prolonged blood coagulation time to the normal range within one hour.



TABLE II

EVALUATION OF THE NECESSARY AMOUNT OF VITAMIN K GIVEN INTRAMUSCULARLY TO REDUCE THE BLOOD COAGULATION TIME TO NORMAL IN CHICKS WITH FOWL HEMOPHILIA

BIRDS	AGE DAYS	INITIAL COAGULATION TIME	VITAMIN K AMOUNT	COAGULATION TIMES AFTER INJECTION		
				1 HR.	6 HR.	24 HR.
<i>Group IV</i>						
XI 48	28		0.27 mg.			8'
XI 103	28		0.27 mg.			7'
XI 109	28		0.27 mg.			29'
XII 114	21	94'	0.27 mg.	32'	22'	
XII 115	21	46'	0.27 mg.	7'	7'	
XII 116	21	6 hours	0.27 mg.	8'		
XII 117	21	33'	0.27 mg.	28'	10'	
XII 118	21	13'	0.27 mg.	10'	5'	
XII 119	21	10'	0.27 mg.	4'	4'	
XII 120	21	6 hours	0.27 mg.	12'	12'	
XII 121	21	46'	0.27 mg.	5'	6'	
<i>Group V</i>						
XII 151	26	48'	0.135 mg.	6'		
XII 152	26	> 5 hours	0.135 mg.	Dead		
XII 153	26	> 5 hours	0.135 mg.	32'		
XII 154	26	> 5 hours	0.135 mg.	77'		
XII 155	26	> 5 hours	0.135 mg.	54'		
XII 156	26		0.135 mg.	22'		
XII 157	26		0.135 mg.	8'		
XII 158	26		0.135 mg.	11'		
XII 159	26		0.135 mg.	33'		

Table II records the blood coagulation times in 20 chicks receiving small doses of vitamin K by injection, 11 birds in Group IV receiving 0.27 mg., and 9 in Group V receiving 0.135 mg. Initial times were taken in 13 instances, all but 2 being definitely prolonged. The findings in Group IV show (1) that a dose of 0.27 mg. is effective in twenty-four hours (3 birds); (2) that it is also effective in one hour (8 birds); and (3) that the average coagulation time was only slightly shorter six hours after injection than at the one-hour period (7 birds). The average time at one hour was fourteen minutes, and at six hours was nine minutes; an apparently definite reduction appearing in 4 of 7 birds. In one bird (XII-116) the blood taken at the six hours period failed to coagulate, presumably due to a technical error. In Group V, 0.135 mg. of vitamin K proved effective in reducing the blood coagulation time within one hour, but in only half the cases was it reduced to less than thirty minutes. In the first 5 birds of the group initial coagulation times were taken, and in 4 these were greater than 5 hours, consequently marked reductions occurred, even though normal figures were not always reached. One bird died of shock following the injection. Of the 4 birds not bled before giving vitamin K, 3 showed a time under thirty minutes (22, 8, and 11) one hour after injection, and the fourth was only thirty-three minutes, so that it seems improbable that bleeding alone could have materially altered the times in the first 4 birds. The results of these experiments indicate that the minimum dose of vitamin K given intramuscularly, which is highly effective within one hour, lies between 0.135 mg. and 0.27 mg.

The injection of very large doses of vitamin K intramuscularly in chicks is apparently fatal. Two birds receiving 54 mg. (0.2 c.c.) died in convulsions

within sixty seconds. Four other birds received 27 mg (0.1 cc) which made them very restless and excited. One of them died in violent convulsions ten minutes later.

The effect of hemorrhage on the coagulation time of chick's blood is extremely important and must be considered in every experiment, as the loss of even a small amount of blood may greatly reduce the time within a short period of observation. This has been recognized during the three years that the writer has been studying the blood of chickens, consequently, the series of experiments recorded in Table III and Fig 1 were devised to try to obtain some accurate information on this subject. Group VI in this table is comprised of eight 23 day-

TABLE III

THE EFFECT OF BLOOD LOSS UPON THE COAGULATION TIME OF CHICKS MARKED REDUCTION  
WITHOUT THE ADMINISTRATION OF VITAMIN K

BIRDS	AGE DAYS	COAGULATION TIME BEFORE		COAGULATION TIME
		INITIAL	BLEEDING	1 HR. LATER
<i>Group VI</i>				
XII 78	23	248		37
XII 75	23	>5	hours	17
XII 80	23	245		17
XII 74	23	124		30
XII 76	23	>5	hours	13
XII 72	23	>5	hours	2 hours
XII 79	23	>5	hours	2 hours
XII 73	23	>5	hours	45
<i>Group VII</i>				
21	60	62		70
22	60	24	hours	24 hours
23	60	6	hours	12 hours

old chicks, all of which showed very prolonged coagulation times at the initial bleeding. Contrary to the procedure employed in the previous groups, no effort was made to control bleeding in these birds, so that most of them oozed blood continuously during the hour before the coagulation time was again tested. None died however. In contrast to this group, the 3 older (2 months) and larger (600 gm, 490 gm, and 520 gm) birds of Group VII were initially bled only the 5 drops usually obtained for the coagulation test, and then had continuous pressure applied to the incised wing vein until oozing of blood ceased. They were also retested at the end of one hour. As the table shows, the smaller birds which bled freely, losing a proportionately larger amount of blood than the older birds, all showed a marked reduction in their blood coagulation times, and in half the cases this reduction was actually down within the normal range, although beyond the average normal findings. The 3 larger birds, losing very little blood, showed no reduction in their prolonged coagulation times. It is obvious that there is a quantitative relationship between the amount of blood loss and the degree of reduction of an abnormal coagulation time, and that a very small blood loss will not alter the time, while a large loss may reduce it to normal even within the period of an hour.

Fig. 1 records graphically the reductions in coagulation times at ten-minute intervals for an hour following the injection of 1.35 mg. of vitamin K into 2 chicks, as compared to the same experiment in a chick which received no vitamin. Every effort was made to staunch hemorrhage between bleedings. The course of events is similar in both experiments, with times of thirteen minutes or less at the end of thirty minutes and of six minutes or less at the end of one hour. The reduction to a five-minute time, ten minutes after receiving vitamin K in one chick, may be due to a technical error, but a repetition of this experiment in another chick showed a reduction from an initial coagulation time of over two hours to sixteen minutes, ten minutes after the injection of 1.35 mg. of vitamin K, with a rise to twenty-eight minutes, twenty minutes after the injection.

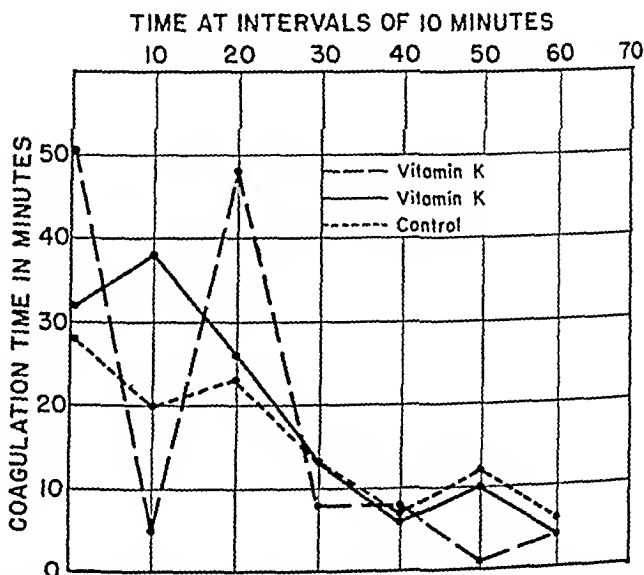


Fig. 1.—Comparison of the effect on the blood coagulation time of initial injections of vitamin K in two chicks to one control without vitamin K. Similar results.

The time was only two minutes at the end of one hour. Here again the effect of blood loss in lessening prolonged coagulation time in the chick is astonishingly similar to that produced by the injection of a large amount of vitamin K.

*The Injection of Vitamin K in Man.*—The same preparation of vitamin K used in chicks was injected intramuscularly into the right upper arm of a hemophilic patient in a dose of 1.0 c.c. two weeks after it had last been shown to be active in chicks. It was autoclaved the day before using it, which concentrated it to about one-half its original volume, 1.0 c.c. containing approximately 0.5 gm. The injection produced moderate tenderness and induration, but no systemic reactions. As Fig. 2 shows, the blood coagulation times were tested before the injection and hourly for three hours thereafter, then at six hours, and then daily for four days. No significant change in the time of coagulation took place, although it was only one hour on the second day compared to an initial time of two hours and forty minutes. During this experiment the activity of the vitamin was again assayed against chicks and was found to be completely inactive. It was then discovered that the entire supply of vitamin K had in-

advertently been left out of the icebox by the technician for several days before this experiment was started. This may have caused its loss of potency.

As a fresh supply of active vitamin K became available for use a month later, through the courtesy of the Abbott Laboratories, the experiment was repeated using the first observations as a control. This time a dose of 100 mg of vitamin K in 1 cc of sesame oil was given to the same patient intramuscularly.

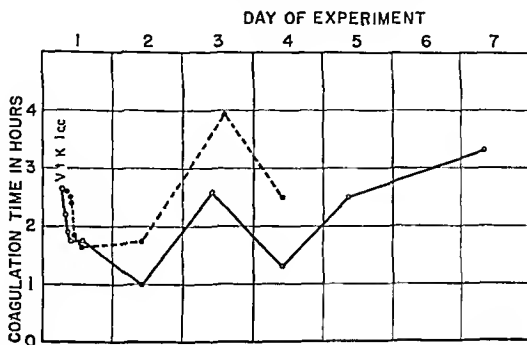


Fig. 2.—Blood coagulation time studies on a hemophiliac receiving two separate injections of vitamin K. In the longer experiment the vitamin was probably inactive. No beneficial effects.

This dose was selected on the basis, that if a 100 gm chick required 0.2 mg, a 50 kg patient would require 500 times this amount or 100 mg, providing a body weight dosage basis in birds can be applied to human beings. No untoward local or general reactions occurred following this intramuscular injection of vitamin K, or following twelve others given at later dates. Fig. 2 shows that the blood coagulation times, followed as in the first experiment were of approximately the same magnitude, and that no significant change in time took place over a period of four days. After this experiment the vitamin preparation used was assayed and found to be active at about a 50 mg per kg of diet level.

It may be concluded from this experiment that vitamin K may be safely injected into man in the same proportionate dose as in chicks, but that no rapid reduction in the blood coagulation time occurs in human hemophilia as in vitamin K deficiency in fowls. However, in a subsequent experiment in this same patient the coagulation time was reduced to normal (ten minutes) within the ten days following the injection of 0.7 mg of vitamin K in sesame oil. As this patient's blood had been examined a number of times over a period of seven and one half years, and the coagulation time had never previously been less than one hour, it is hardly likely that the development of a normal coagulation time for the first time was only coincidental. The details of this experiment, together with other similar ones, will be reported elsewhere.

#### DISCUSSION

Vitamin K deficiency in chicks known as fowl hemophilia may be readily produced in the laboratory, providing strict attention is paid to the type of diet

used and the housing conditions of the birds. Once the deficiency is produced in a large number of chicks, it is possible not only to study the nature of the blood dyscrasia, but also the effect of vitamin K upon it. It has been shown in Tables I and II that the hypodermic injection of this vitamin intramuscularly is highly effective in a dose as small as 0.135 mg. per chick of about 100 gm. of weight, and that the reduction in the blood coagulation time takes place within the period of one hour. It is nontoxic in relatively small doses in chicks and in man.

Great care must be exercised not to confuse the effect of hemorrhage in reducing the blood coagulation time with the similar effect produced by the administration of vitamin K, as has been shown in Table III and Fig. 1. An appreciation of this fact is of the utmost importance in interpreting the results of vitamin K therapy when an initial coagulation time has been obtained, as a *reduction in the time may be due either to the initial bleeding or to the vitamin*, even when a relatively very small amount of blood has been removed. An exact quantitative relationship has not yet been determined, but if only a few drops of blood are shed and subsequent bleeding is well controlled, hemorrhage alone is probably not the sole cause of a marked reduction in the blood coagulation time. It should also be noted that once birds on a diet deficient in vitamin K have been bled and have survived, the blood coagulation times may remain normal for weeks, rendering the chicks useless for further experiments. Five chicks with prolonged coagulation times rebled three weeks later all showed normal times which ranged from one to thirteen minutes.

The truly remarkable effect of vitamin K in reducing the blood coagulation time of chicks from many hours to a few minutes within one hour after a single injection is paralleled by an extraordinary clinical observation. Birds with fowl hemophilia tend to bleed continuously after the wing vein is incised, and not infrequently succumb from fatal hemorrhage within one or more hours. If these birds are promptly injected with adequate doses of vitamin K after the initial bleeding, *hemorrhage from the wing vein stops almost immediately*. This was observed again and again, and at the time the 8 birds were bled in Group VI, a similar control group was also run, the only difference being that these birds received injections of vitamin K directly after the initial bleeding. In this control group not only was there not any gross blood on the floor of their cage one hour later when the second bleeding was performed, but the wings were not spotted with blood, and the previously incised veins were covered with clot. Of the birds in Group VI which received no vitamin K, all showed evidence of free bleeding at the end of the hour, 2 were almost moribund, and the floor of their cage was covered with blood. Some of the wing veins showed no evidence of blood clot. Apparently vitamin K produces an almost immediate effect on the blood vessel wall or on the surrounding tissue juices which tends to arrest hemorrhage, even before any constant reduction in blood coagulation time can be demonstrated.

The nature of the blood coagulation defect in fowl hemophilia has been designated as a quantitative defect in prothrombin by Quick.<sup>10</sup> This investigator's data and the interpretation thereof are certainly open to question. He

studied relatively few birds on a deficient diet, the coagulation times of these birds were actually normal (under thirty minutes) in most instances, and *specific* data on normal control birds in his series are conspicuously lacking. He has not considered the effect of hemorrhage on either the coagulation time or the prothrombin content of the blood. Although the amount of prothrombin (thermolabile globulin) is apparently deficient in these birds, which is in accord with the observations of Cheney and Rantz<sup>11</sup> in studying this problem, *there is no positive evidence whatsoever that a deficiency in prothrombin is in itself the specific cause of the blood coagulation defect.* The almost explosively beneficial effect of an injection of vitamin K can hardly be explained on the basis of a sudden flooding of the blood stream from some hidden source with a special form of globulin, particularly when the blood loss from the initial bleeding in some birds must deprive the bird of considerable blood globulin rather than tend to increase it. A marked loss in globulin in chicks with fowl hemophilia may well be part of a general diminution in blood proteins dependent on their restricted diet, on blood loss, or on some alteration in their metabolism. As no complete blood protein studies on chicks are available this question cannot yet be settled. As long as the nature of the normal blood clotting mechanism remains in doubt, it is most difficult to interpret the abnormal, but it seems possible that the relationship of vitamin K to blood clotting may throw some new light on this whole subject.

The application of the findings in fowl hemophilia to blood dyscrasia problems in human beings is beyond the scope of this report except where it has been shown that the parenteral administration of vitamin K to patients is feasible, and that no response to the injection of this vitamin occurs in human hemophilia comparable to that in the chick. In 1936 the writer found that feeding a vitamin K rich diet to 2 patients with hemophilia was without effect, and this has been reported by Dam,<sup>12</sup> but the case herein reported is apparently the first to be treated by injections of this vitamin. A number of papers are appearing in the literature<sup>9, 13, 14</sup> stating that hemorrhage in jaundice is due to a quantitative prothrombin defect dependent on a deficiency in vitamin K, but there is at present very little basic work to establish such a relationship so that clinicians should be most wary of using vitamin K therapeutically, except on purely experimental grounds.

#### CONCLUSIONS

- 1 A single intramuscular injection of vitamin K in chicks with a prolonged blood coagulation time due to a diet deficient in vitamin K (fowl hemophilia) will reduce the time to normal within the period of one hour.
- 2 The minimum effective dose in one hour by injection is between 0.135 mg and 0.27 mg in three week old chicks, weighing approximately 100 gm.
- 3 Blood loss may also reduce the prolonged coagulation time of fowl hemophilia to normal.
- 4 The intramuscular injection of vitamin K in man is apparently a safe procedure. No certain indications for its use have yet been established, but it may prove of value in cases of jaundice which tend to bleed.

## REFERENCES

1. Dam, H.: Cholesterinstoffwechsel in Huhnereierein und Huhnchen, *Biochem. Ztschr.* 215: 475, 1929.
2. Idem: Hemorrhages in Chicks Reared on Artificial Diets, New Deficiency Disease, *Nature* 133: 909, 1934.
3. Almquist, H. J., and Stokstad, E. L. R.: Hemorrhagic Chick Disease of Dietary Origin, *J. Biol. Chem.* 111: 105, 1935.
4. Idem: Factors Influencing the Incidence of Dietary Hemorrhagic Disease in Chick, *J. Nutrition* 12: 1936.
5. Almquist, H. J.: Anti-hemorrhagic Vitamin, *Poultry Science* 16: 166, 1937.
6. Idem: Crystals With Vitamin K Potency, *Nature* 140: 25, 1937.
7. Idem: Assay Procedure for Vitamin K (Anti-hemorrhagic Vitamin), *J. Nutrition* 14: 329, 1937.
8. Idem: The Quantitative Determination of Vitamin K, *Biochem. J.* 30: 890, 1937.
9. Dam, H., and Glavind, J.: Vitamin K in Human Pathology, *Lancet* 234: 720, 1938.
10. Quick, A. J.: The Coagulation Defect in Sweet Clover Disease and in the Hemorrhagic Chick Disease of Dietary Origin. A Consideration of the Source of Prothrombin. *Am. J. Physiol.* 118: 260, 1937.
11. Cheney, Garnett, and Rantz, Lowell: To be published.
12. Dam, H., Schonheyder, F., and Lewis, L.: The Requirement for Vitamin K of Some Different Species of Animals, *Biochem. J.* 31: 22, 1937.
13. Snell, A. M.: Symposium on Hemorrhagic Diathesis in Cases of Jaundice: Relation to Vitamin Deficiency; Preliminary Report, *Proc. Staff Meet., Mayo Clin.* 13: 65, 1938.
14. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: Bleeding Tendency of Obstructive Jaundice, Prothrombin Deficiency and Dietary Factors, *Proc. Soc. Exper. Biol. & Med.* 37: 628, 1938.
15. Quick, A. J.: The Nature of Bleeding in Jaundice, *J. A. M. A.* 110: 1658, 1938.

210 POST STREET

## LYMPHOSARCOMA OF THE MEDIASTINUM (MALIGNANT THYMOMA)\*

### A CLINICAL AND PATHOLOGICAL STUDY WITH CASE REPORT OF A CHILD

EDWARD J. BOMZE, M.D., AND JACK D. KIRSHBAUM, M.S., M.D.  
CHICAGO, ILL.

**T**UMORS in the mediastinum originating in the region of the thymus gland are comparatively rare, especially in children. In a series of 10,800 consecutive autopsies performed at the Cook County Hospital from January, 1929, to June, 1938, four cases of malignant thymoma were encountered, an incidence of 0.036 per cent. One of these cases, the one here reported, occurred in a child. Symmers<sup>1</sup> in 1932 reported 25 cases in a series of 17,000 autopsies, an incidence of 0.14 per cent, while Crosby<sup>2</sup> in the same year reviewed 166 cases from the literature (including his own case) of malignant thymoma.

#### PATHOLOGY

There is some controversy among authors as to the classification and nomenclature of malignant thymic tumors. All the classifications proposed are

\*From the Department of Pathology, Cook County Hospital, Chicago.  
Received for publication, December 6, 1938.

based on the essential cell type or on the thymic constituents from which the tumor originates. The simplest and most generally accepted one is that of Ewing,<sup>2</sup> who subdivides these tumors into three groups as follows: (1) Lymphosarcoma, which is composed of small round cells and arises from the small thymic lymphocytes or thymocytes. This is the most frequent type of tumor which usually occurs in children, and is characterized by a limited tendency to metastasis, usually to neighboring structures. (2) Carcinoma arising from the epithelial cells of the thymic reticulum and occurring in individuals past 40 years. These resemble grossly the lymphosarcoma but are markedly radio-resistant. (3) Spindle cell sarcoma, in which the tumor takes its origin from the scanty connective tissue stroma of the thymus. This type is very rare and occurs in individuals under 40 years.

Andrus and Foot<sup>3</sup> classify thymomas as nonmalignant and malignant, subdividing the malignant forms more strictly on the basis of basic cell form into the following seven groups: (1) thymocytic or lymphocytoid type, (2) large celled or lymphoblastic type, (3) thymic reticulum cell type, (4) perithelial type, (5) granulomatous type, (6) epithelial or carcinomatous type, and (7) teratoid type.

Evans,<sup>4</sup> in discussing his case proposes a classification similar to that of Ewing, but in addition subdivides the lymphosarcomatous form into two types, first, those which are composed of masses of lymphoid tissue separated by connective tissue strands and, secondly, those tumors in which there is a resemblance to Hodgkin's disease showing giant cells and eosinophilic leucocytes.

Ewing's classification to us is the simplest and most logical one. Most authors, however, agree on one point, that the thymoma histologically is characterized by the marked polymorphism of the cells and the great variety of structural forms.

#### Symptoms

The clinical picture is not absolutely characteristic but if the condition is kept in mind, an ante mortem diagnosis is not difficult. The onset of symptoms, which occurs early in the course of the disease, may be ushered in gradually or suddenly. The most common symptoms are those of compression, such as cough, orthopnea, attacks of dyspnea, and hoarseness. A swelling may appear in the lower part of the neck, with a sense of oppression beneath the sternum, and in a few cases substernal pain is noted. Later the effects of compression predominate the clinical picture, causing cyanosis, distention of the superficial chest veins, edema of the face, neck, and occasionally of an arm. Respiration becomes visibly difficult, and an area of dullness beneath the upper half of the sternum occurs. Fluid may appear on one or both sides, and there may be signs of compression of one or both apices of the lungs. When the tumor becomes large, there may be a difference in the pulse and blood pressure between the two upper extremities.

The x-ray and fluoroscopic findings are very characteristic and reveal a widening of the upper mediastinal shadow. A biopsy may be taken if the tumor presents itself in the neck in order to establish or confirm the diagnosis.

The prognosis of thymic tumors is poor. However, several cures have been reported. Since the lymphosarcomatous type is radiosensitive, radium has been employed, and Cutler<sup>5</sup> reports a case clinically well seven years after the onset



of symptoms. Cutler states, "Usually those tumors which respond rapidly are so highly malignant that they are accompanied by a grave prognosis"

Of the 4 patients with thymic tumor encountered in our material, 3 were of the lymphosarcomatous type, ages 58, 53, and 12, respectively, and one was a carcinoma in an individual aged 37. Because of the rarity of malignant thymomas in children, we are reporting the following case.

#### CASE REPORT

The patient, aged 12, was brought to the Cook County Hospital, April 19, 1938, suffering from extreme dyspnea and cyanosis. He died while being examined. The mother stated that he had been at the Children's Memorial Hospital for the same trouble several weeks previously and had been discharged improved on April 11, 1938. The history, as obtained through the kind courtesy of the Children's Memorial Hospital, was as follows: The patient, a white boy of Polish descent, was brought to their dispensary on March 27, 1938, at which time the mother stated that the boy had been perfectly well until six days previously (March 21, 1938). At that time he began to cough slightly and had much difficulty in breathing. A swelling in the anterior and lower part of his neck became visible. A physician was called on the morning of March 27, 1938, the sixth day of symptoms, and his illness was diagnosed as diphtheria.

The patient was immediately taken to the Municipal Contagious Hospital and there given diphtheria antitoxin. Following the administration of the antitoxin, an x-ray examination was made; this showed a mass in the chest. The patient was then referred to the Children's Memorial Hospital. By this time he was very dyspneic and somewhat cyanotic.

*Physical examination* revealed a severely dyspneic, somewhat cyanotic, white male child in acute respiratory distress. There was a large, nontender, sharply circumscribed mass in the thyroid area of the neck. Two large dilated veins in the skin of the chest were seen to come from under the sternoclavicular region and pass up to the chin. There was dullness, especially to the left of the sternum and to the left of the spine, with some flatness. Breath sounds were suppressed in these areas. There was an inspiratory and slight expiratory stridor. Temperature and blood pressure readings were normal. The boy appeared well nourished and well developed.

*Laboratory Examination:* Urine was entirely negative, the tuberculin skin tests were negative. The Wassermann, Kahn, and Kolmer tests were negative. Blood examinations were done on numerous occasions because of the strong possibility of leucemia. The blood findings are noted in Table I.

TABLE I  
BLOOD WORK

DATE	HB	RBC	WBC	DIFFERENTIAL COUNT				PLATELETS
				POLY	LYMPH	MONO	BAZO	
	<i>Per cent</i>				<i>Per cent</i>			
3/27/38	80	4,080,000	24,800	84	12	4		261,000
3/28/38			14,800	77	18	4	1	
3/30/38			9,100	48	52			
X-ray Rx								
3/31/38	90	4,700,000	5,600	54	45		1	
4/11/38			6,100	87	12	1		

A sternal bone marrow puncture revealed in 500 cells counted: lymphocytes 30.2 per cent; segmented neutrophils 29.6 per cent; stab neutrophils 21.4 per cent; metamyelocyte 8 per cent; neutrophilic myelocytes 6.6 per cent; eosinophilic myelocytes 0.2 per cent; blast forms 1 per cent; mature eosinophils 0.2 per cent; monocytes 2 per cent; erythroid nucleated elements 0.8 per cent.

An x-ray examination of the chest revealed a dense tumor mass in the upper mediastinum, predominantly on the left side and anterior to the trachea. The lungs were hyperaerated, and there was accentuation of the bronchial markings below each lung hilus.

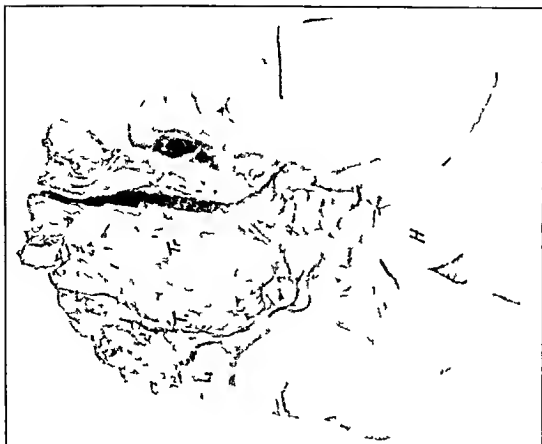


Fig 2

Fig 2—Photograph shows the tumor (T) cut longitudinally compressing the trachea (Tr) and extending downward over the pericardial sac and heart (H)



Fig 1

Fig 1—Photograph shows the tumor (T) cut longitudinally compressing the trachea (Tr) and extending downward over the pericardial sac and heart (H)

Fig 2—Photograph shows the tumor posteriorly compressing the apex of the left lung and covering the left side of the trachea (T = tumor, L = lung, H = heart, Tr = trachea)

Chalcal diagnosis was not definitely made. The predominating opinion, however, was that the patient was suffering from (1) lymphatic leucemia (aleukemic phase), with formation of a mediastinal mass or (2) from a mediastinal tumor of unknown origin.

**Course.** On March 30 following x-ray therapy (190 roentgen units for five minutes), the tumor mass decreased in size about 40 per cent and there was less emphysema of the lungs. However, when another x-ray film was taken on April 9, ten days later the tumor shadow was 1 cm wider than on the previous examination but 3 cm narrower than when first visualized. The patient was discharged from the hospital April 11, 1938, frequently

returning to the out-patient dispensary. He remained well for a week, and then suddenly developed respiratory distress and cyanosis. He was rushed to the Cook County Hospital and died while being examined.

*Post-Mortem Findings:* The autopsy was performed one and one half hours after death. There was a marked cyanosis about the head. The essential pathologic findings were confined to the thorax. The left pleural cavity contained about 150 em. of a clear yellow fluid. The anterior mediastinum was completely filled by a large, firm, rubbery tumor mass, measuring 17 cm. in vertical diameter, 10 cm. in transverse diameter and 10 cm. in anteroposterior diameter. The mass was adherent to the pericardial sac, extending downward to the apical region, and upward to the level of the thyroid cartilage. It slightly compressed the apex of the left upper pulmonary lobe and the left main bronchus, and completely encircled the trachea, thereby compressing its lumen (Fig. 1). On the posterior aspect of the trachea the tumor formed a plate, measuring 8.5 by 4.5 cm. (Fig. 2). On sectioning the mass was pale gray tan in color, irregularly lobulated, and mottled with pinhead to 2 cm. in diameter dark purple red areas.

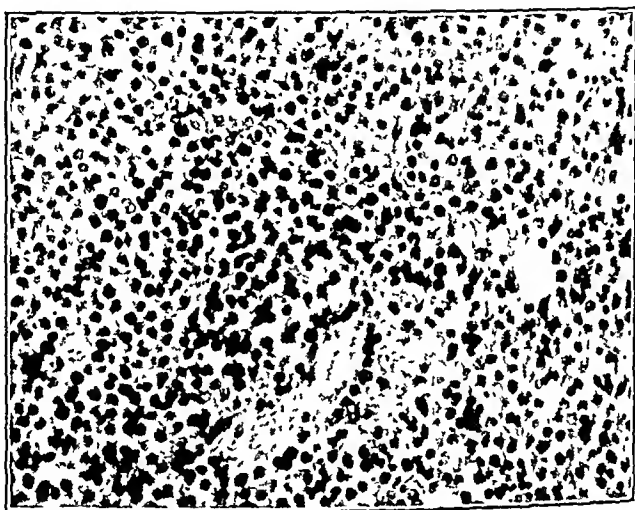


Fig. 3.—Photomicrograph of the tumor showing the lymphocytic character of the type cell (Magnification  $\times 300$ .)

The bifurcation lymph nodes were enlarged up to 20 mm. in diameter, firm, and although matted together, their individual outlines were still discernible. On sectioning, the surface was purple gray with multiple hemorrhagic areas scattered throughout. The mucosa of the trachea and main bronchi was covered by a frothy blood-stained mucus.

The left lung was subeipitant throughout, and on the anterior surface there were numerous hemorrhagic areas up to 8 mm. On sectioning, the cut surface was uniformly deep purple gray and moist with blood. The right lung was similar in appearance. The other findings showed nothing unusual.

*Microscopic Examination:* Sections from the tumor in the mediastinum showed a uniform and rather monotonous picture made up of a diffuse proliferation of small cells of the lymphocyte type (Fig. 3). Their nuclei for the most part filled the cell and there was a very narrow rim of cytoplasm. One cell bordered on another and was frequently separated by fine fibrils which stained steel blue with Azon stain. No mitotic figures were seen. No clear-cut Hassall's bodies were seen in these sections, but in many places single isolated epithelial cells, reticulum cells, and structures resembling fragmented degenerated portions of Hassall's corpuscles could be seen.

Sections of the spleen revealed large lymph follicles with large germinal centers containing single swollen mononuclear cells, and single polymorphonuclear leucocytes. The sinuses appeared markedly dilated and contained much blood.

*Anatomic Diagnosis* Lymphosarcoma of the anterior mediastinum (malignant thymoma), compression of the apex of the left lung the heart, and the trachea, left hydrothorax, edema and passive congestion of both lungs, passive congestion and follicular hyperplasia of the spleen, parenchymatous degeneration of the heart liver and kidneys

#### DISCUSSION

The diagnosis of a malignant thymoma in our patient is suggested in view of the anatomic location of the tumor mass and the typical histologic picture. Although typical Hassall's corpuscles which are characteristic of the thymus gland were not found, there were structures suggesting remnants of the gland. That we were not dealing with a sarcomatous transformation of a lymphatic leucemia is evidenced by the absence of a generalized or localized lymphadenopathy, the normal blood picture, and the absence of leucemic infiltrations in the internal organs. Clinically the tumor showed signs of being radiosensitive, and the rapid death of the patient was caused by compression of the trachea and heart.

According to Jones<sup>6</sup> and Brown, the thymic origin of the mediastinal sarcomas is indicated by the presence of a large slightly lobulated tumor at the site of the thymus, which extends downward behind the sternum without infiltration of bone, involves the pericardium and pleura by direct extension, and histologically resembles thymic tissue. Crosby<sup>8</sup> adds to these criteria the tendency of thymic tissue to surround and compress the trachea bronchi, pericardium, and the great vessels. Death is caused by compression of the air passages, and less often by invasion of the vessels thus producing asphyxia and venous obstruction which may develop gradually or very suddenly.

From a clinical point of view the recognition of thymic tumors particularly those of the lymphocytic type, is important in spite of the infrequency of their occurrence. The tumor usually indicates its presence comparatively early in its course of development because of its location and proximity to the trachea, bronchi, great vessels, lungs, and heart. The characteristic radiosensitivity of these tumors offers hope of cure, or at least spectacular relief, of the extremely distressing respiratory difficulty and obstructive manifestations, with consequent prolongation of life in relative comfort. Also, several cases have been reported in which complete surgical removal of the tumor was successfully accomplished, resulting in complete cures the patients being alive and well eight months to two years or more following operation.

According to Crosby,<sup>8</sup> cough or hoarseness without expectoration or hemoptysis is usually the first symptom in those cases in which the onset is gradual. Dyspnea then becomes apparent or may be the initial symptom in cases in which the symptoms come on suddenly. Other less frequent symptoms are puffiness about the eyelids, fullness in the anterior part of the neck, and a pink discoloration of the eyelids. Later there is engorgement of the veins of the neck, evanosis edema of the face and upper extremities, palpitation, substernal pain and oppression, dysphagia, headaches, and progressive severe dyspnea. In some cases inequality of the pulses in the upper extremities is found. Pleural effusions, most commonly on the left side, hydrothorax, and cardiac displacement are seen in many cases.

The most important diagnostic measure is the x-ray examination of the chest. The findings are very suggestive, if not absolutely characteristic, and in the opinion of many roentgenologists an absolute diagnosis can be made providing the condition is kept in mind. Doub<sup>9</sup> describes the x-ray findings as consisting of a "more or less circular sharply-defined, non-pulsating shadow just above the cardiac shadow in the position ordinarily occupied by the thymus gland." X-ray films in the lateral position reveal the tumor occupying the anterior mediastinum. The mass may project to one side and may not be symmetrically placed. In some cases it is irregular in outline. Fluoroscopic examination is valuable in ruling out the pulsations of an aortic aneurysm. In some advanced cases there may be extension into the pleura or lungs, with haziness of the lung fields so that the circumscribed tumor cannot be seen.

Many cases have been reported by Norris,<sup>10</sup> Kahr,<sup>11</sup> Craver and MacComb,<sup>12</sup> Margolis,<sup>13</sup> and Foot and Harrington,<sup>14</sup> in which a thymoma was associated with myasthenia gravis, Addison's disease, and osteomalacia.

The differential diagnosis includes such conditions as aortic aneurysm, mediastinal abscess, leucemia, chronic mediastinitis, substernal thyroid, Pott's disease, tuberculous mediastinal lymph nodes, generalized lymphosarcoma, Hodgkin's disease, and intrathoracic goiter as described by Kirshbaum and Rosenblum.<sup>15</sup>

#### SUMMARY

A case of lymphosarcoma in the region of the thymus gland, or so-called malignant thymoma, in a child is here presented. In a review of 10,800 necropsies, 4 cases of malignant thymoma were encountered, 3 cases of the lymphocytic type (lymphosarcoma), and one of the epithelial type (carcinoma). The differential diagnosis of intrathoracic lesions is discussed, and the pathologic and clinical manifestations of malignant thymoma are emphasized.

#### REFERENCES

1. Symmers, D.: Malignant Tumors and Tumor-like Growths of the Thymic Region, *Ann. Surg.* 95: 544, 1932.
2. Ewing, James: Neoplastic Diseases, ed. 3, Philadelphia, 1928, W. B. Saunders Co.
3. Andrus, W. Dew, and Foot, N. C.: Report of a Large Thymic Tumor Successfully Removed by Operation, *J. Thoracic Surg.* 6: 648, 1937.
4. Evans, J. H.: Malignant Tumor of the Thymus Gland, *Brit. M. J.* 1: 19, 1938.
5. Cutler, M.: Lymphosarcoma, Clinical, Pathological and Radiotherapeutic Study With Report of 30 Cases, *Arch. Surg.* 30: 445, 1935.
6. Jones, A. C.: Lymphosarcoma of the Thymus, *Tr. Am. Laryng., Rhin. & Otol. Soc.* 34: 478, 1928.
7. Brown, S. E.: Malignant Tumor of the Thymic Region, *Arch. Path. & Lab. Med.* 2: 822, 1926.
8. Crosby, E. H.: Malignant Tumors of the Thymus Gland, *Am. J. Cancer* 16: 461, 1932.
9. Doub, H. P.: Roentgen Diagnosis and Treatment of Thymomata, *Radiology* 14: 267, 1930.
10. Norris, E. H.: A Thymoma (Adenoma of Thymus) From an Unusual Case of Myasthenia Gravis, With Observations on the General Pathology, *Am. J. Cancer* 30: 308, 1937.
11. Kahr, H.: Carcinoma of the Thymus Gland in a Five Month Pregnant Woman, *Zentralbl. f. Gynäk.* 60: 1751, 1936.
12. Craver, L. F., and MacComb, W. S.: Lymphatic Leukemia With Thymic Enlargement, *Am. J. Cancer* 16: 277, 1932.
13. Margolis, H. M.: Tumors of the Thymus, *Am. J. Cancer* 15: 2106, 1931.
14. Foot, H. C., and Harrington, H.: Malignant Epithelial Thymoma, *Am. J. Dis. Child.* 26: 1264, 1923.
15. Kirshbaum, J. D., and Rosenblum, A. H.: Suppurative Intrathoracic Thyroiditis, *Arch. Surg.* 36: 867, 1938.

## EXPERIMENTAL NEPHRITIS PRODUCED BY STAPHYLOCOCCUS TOXIN IN THE DOG\*

R. H. RIGNON, M.D. NASHVILLE TENN.

NECROSIS in the cortex of the kidney may follow the intravenous injection of staphylococcus toxin in the dog and rabbit. All investigators do not agree as to the pathogenesis of this lesion. Neisser and Levaditi<sup>1</sup> in 1900 first described it in the rabbit and expressed the opinion that emboli formed by degenerating leucocytes obstruct the smaller blood vessels and infarcts subsequently develop. Rigdon and his associates in 1934 concluded from their studies that the cortical necrosis was the result of direct injury to the epithelium and endothelium of the glomeruli and the epithelium of the tubules. Von Glahn and Weld<sup>2</sup> in 1935 noted an early necrosis of some of the renal arteries and stated that "the changes in the vascular elements of the kidney were further advanced than those in the epithelium of the tubules. When the arteries were occluded by fibrin the circulation must have ceased through certain portions of the cortex and it would seem most plausible that this occlusion determined in many of the kidneys the widespread cortical necroses that are indeed of the nature of infarcts."

Glynn<sup>3</sup> in 1937 expressed the opinion that staphylococcus toxin "causes a dilatation of small arteries, arterioles and capillaries. It is excreted through the glomeruli and causes direct damage to tubular epithelium. It injures capillaries, produces loss of tone with subsequent extreme dilatation and eventually sudden rupture. The sudden bursting of glomerular loops is the mechanism by which hemorrhage occurs. Necrosis quickly follows for the tubular epithelium already damaged by the toxin has little resistance to the anemia resulting from the hemorrhage."

This report is a study of the renal lesions and their pathogenesis in the dog following the intravenous and intrarenal arterial injection of staphylococcus toxin. The latter route of inoculation was used in an attempt to increase the concentration of toxin in the kidney.

### MATERIAL AND METHODS

The staphylococcus toxin was prepared by growing a hemolytic *Staphylococcus aureus* in veal infusion broth for forty eight hours. The toxin was filtered through a Berkefeld candle, tested for sterility, and stored at 5° C. The technique for producing toxin is given elsewhere.<sup>4</sup> All the toxin contained a hemotoxin. No attempt was made to standardize it on the basis of renal necrosis, since this problem was one to study the lesions produced by this toxin.

\*From the Department of Pathology, Vanderbilt University Medical School, Nashville.  
Received for publication December 9, 1938.

TABLE I

DATA, AS SHOWN BY 6 DOGS, INDICATING THE TIME AND THE AMOUNT OF STAPHYLOCOCCUS TOXIN GIVEN INTRAVENOUSLY TO ALL 26 ANIMALS IN THIS GROUP AND THE VARIATIONS OBSERVED IN THE NONPROTEIN NITROGEN\*

DOG	CON TROL	EXPERIMENTAL DAYS														REMARKS
		1	2	3	4	5	6	7	8	9	10	11	15	16		
1 18.3 kg.	T† N+	10	10			40	25	86		150		109	125	120	Killed on sixth day (See Figs. B, 1, 6, and A, 2 for lesions)	
2 8.25 kg.	T N	6	10 30	33	20 28	20 35	25		35 25	30	20 20		20		Apparently normal on sixteenth day. Discarded	
7 14 kg.	T N	10	20 25	18 38	133	190 240	300 240								Died on sixth day. Extensive renal necrosis	
36 4.3 kg.	T N	2.5	6	12			5 40	75 133							Killed on seventh day. Extensive renal necrosis	
A 12.5 kg.	T N	24													Injected at 2:30 P.M. Dead following morning at 8 A.M. (See Fig. B, 4 for lesions)	
F 13 kg.	T N	10	5	5	10	15									Killed on sixth day. (See Figs. A, 7, B, 2, 3, for lesions)	

\*Six dogs have been included in this series which were injected while I was at Duke University School of Medicine, Durham, N. C. I wish to thank Dr. W. D. Forbus for permitting me to include them in this study.

†T=Staphylococcus toxin in cubic centimeters.

+N=Nonprotein nitrogen in mg. per cent.

Twenty six normal dogs, varying from 0.9 to 18 kg., were given this toxin intravenously, usually in the femoral vein. The frequency and the amount of the inoculum given to the different animals are illustrated by the group of dogs shown in Table I.

Twelve dogs were given staphylococcus toxin directly into the renal artery.\* One of these died on the seventh postoperative day. The other dogs were killed at intervals between the second and sixteenth days of the experiment. The renal vessels in this group of dogs were exposed through an extraperitoneal approach. The toxin was slowly injected into the artery through a 20 gauge needle. Pressure was applied to the artery for approximately three minutes. A ligature was placed under the renal vein and held taut during the injection and for approximately one minute thereafter. The animals were anesthetized with either nembutal or ether.

Staphylococcus toxin heated at 100° C. for two hours was injected into the renal artery of two dogs, and infusion broth was given intravenously to another dog for the control experiment.

All animals were observed closely during the experiment and usually they were autopsied immediately after death. The tissues were fixed in Zenker and 10 per cent formaldehyde solution. Sections were stained routinely with hematoxylin and eosin, and select ones were stained with Mallory's aniline blue connective tissue stain and by Gram's and Giemsa's methods.

The amount of nonprotein nitrogen in the blood was determined before or after the intravenous injection of the toxin in 17 of the dogs.

#### CLINICAL OBSERVATIONS

*Staphylococcus toxin injected intravenously* The dogs react differently. Some vomit, pass small stools, and void five to ten minutes after the inoculation. The vomiting sometimes continues for several hours. Abdominal cramps and tenesmus are often marked. Weakness and general prostration frequently progress until death two to six hours after the inoculation.

The dogs that live for five to six days do not show these acute symptoms. These animals usually vomit and sometimes refuse food and water. Frequently they develop a mild diarrhea with blood in the stools and become very weak. They develop a mild diarrhea with blood in the stools and become very weak. Twelve to twenty hours before death. Groups of muscles frequently twitch during this time. Some dogs apparently become comatose a few hours before death. Convulsions frequently occur during the latter hours of life. Some have involuntary stools, increased salivation, a peculiar stain in their eyes, and fixation of the head or extremities for short intervals. They are too weak to stand at this time, convulsions become more frequent until death.

The dog given only infusion broth did not show any clinical changes different from a normal animal.

*Staphylococcus toxin injected intrarenally* All the dogs in this group except 2 appeared perfectly normal following the inoculation of the toxin. These 2 were sick and refused food on the third day following the operation.

\*Dr. Sanford E. Levy, National Research Fellow in the Medical Sciences, working in the Department of Surgery, injected the renal artery in this group of dogs. I appreciate Dr. Levy's cooperation and wish to thank him and the Department of Surgery for their assistance in this study.





Fig. A—1. Dog F. Pyknotic nuclei, fragmented cells, and few polymorphonuclear leucocytes are present in the tuft. There is an apparent increase in endothelial-like cells in the glomerulus (See Table I, H. & E. X700). 2. Dog 1. There is a proliferation of endothelial-like cells in the tuft about the injured glomerulus (See Table I, H. & E. X550). 3. Dog 3. A diffuse glomerular change. Mononuclear cells are often present about the injured glomerulus (See Table I, H. & E. X200). 4. Dog 4. Only a portion of the glomerulus is affected. Frequent lesion in this dog was injected into the renal artery fifteen days before this dog was killed (H. & E. X200).

One had blood in the stools on the second postoperative day. Both were sick until the fourth and seventh days, at which time one was killed and the other died.

One of the dogs in the control group was normal following the intrarenal injection of the heated staphylococcus toxin; the other refused food and was sick on the ninth day, and was found dead on the morning of the tenth.

*Observations on Nonprotein Nitrogen in Blood.*—Table I shows the amount of nonprotein nitrogen in the blood of 5 of the 17 dogs given staphylococcus toxin intravenously. Three of these dogs lived for forty-eight hours or longer

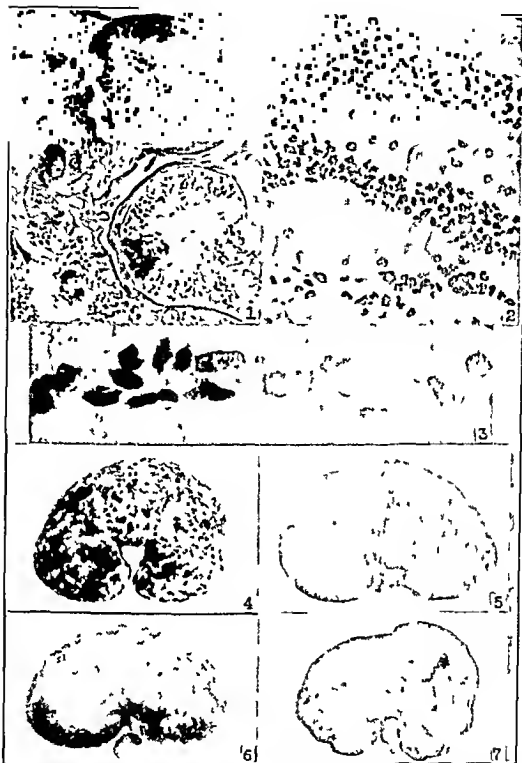


Fig B-1 Dog I Colloid or hyaline droplets are numerous in the tubular epithelial cells (See Table I H & F  $\times 100$ ). 2 Dog F Some of the capillaries between the renal tubules are dilated and filled with blood. 3 Dog F The tubular epithelial cells are severely injured. This dog was killed on the sixteenth day of the experiment. 4 Dog F The tubular epithelial cells lining the capillaries as shown in Fig B-1 are injured. Injury to the endothelium apparently precedes injury to the lumen of the blood vessel. (H & E  $\times 100$ ) 5 Dog F The lesion is bilateral. The kidney was given intravenously at 2:30 P.M. and the dog was found dead at 8:30 A.M. the following morning. 6 Dog 63 This kidney is only slightly smaller than the normal. Petechiae and focal yellow areas are present in the cortex. He was inoculated in the renal artery with 1.5 cc of staphylococcus toxin fifteen days before he was killed. 7 Dog 1 The cortex has many focal pale yellow areas which suggest necrosis. The animal was killed on the sixteenth day of the experiment. (See Table I for the time and amount of toxin given and also the nonprotein nitrogen content of the blood). Dog 62 The greater part of the renal cortex is atrophic. The surface in this area is relatively uniform in appearance. There is one circumscribed area of normal tissue. Apparently no toxin reached this portion of the kidney. 12 cc of staphylococcus toxin was injected into the renal artery sixteen days before this dog was killed.

and showed 120 to 240 mg nonprotein nitrogen at the time of death. Observations were not made on 7 dogs, since they died in less than twenty four hours following the injection of the toxin. The other animals did not show any variation from the normal.

## PATHOLOGIC STUDY

*Staphylococcus toxin injected intravenously.* Usually there are no gross renal lesions in the animals that die during the first six hours following the injection of the staphylococcus toxin. The lesions vary after this time. There are diffuse and circumscribed areas of hemorrhage in some of the kidneys (Fig. B, 4). In others there are petechiae and pale yellow focal areas in the cortex (Fig. B, 6).

The vascular lesions are inconspicuous during the first few hours following the injection of toxin. The endothelial cells lining the glomerular capillaries and the capillary plexus around the renal tubules become swollen, pyknotic, and show karyorrhexis during the first twenty-four hours. Polymorphonuclear leucocytes subsequently accumulate along the wall of some of these capillaries in such numbers that there is apparently some interference in the circulation (Fig. B, 2 and 3). When the injury to the endothelium is less severe and the animal lives for ten days or longer, there are an apparent hypertrophy and hyperplasia of the endothelium. These regenerated cells stain more basophilically than the normal. Some of the capillary loops in the glomeruli are dilated and filled with red blood cells, others show a pink staining coagulated material which apparently represents an accumulation of injured endothelial cells, fibrin, and red blood cells. The basement membranes in such glomeruli also appear disintegrated.

Red blood cells frequently escape through these injured capillary walls. A pink staining albuminous material sometimes fills Bowman's space and apparently compresses the tufts. Some of the glomeruli show polymorphonuclear leucocytes in the capillaries (Fig. A, 1). There is an apparent regeneration and hyperplasia of the glomerular endothelium subsequent to the acute lesion (Fig. A, 2).

The medium and larger renal arteries are normal in all of the dogs, except the one given the staphylococcus toxin intravenously. Many of the vessels in this animal are completely occluded by a friable and necrotic material. There are many focal necrotic areas in the cortex and medulla which apparently have resulted from vascular obstructions.

The lesion in the tubular epithelium is usually pronounced. The cells lining the convoluted portion of the tubule apparently show the most marked change. The earliest lesions, those which occur within the first six hours following the injection of the toxin, are cloudy swelling, pyknosis, karyorrhexis, and rupture of cell membranes. Sometimes the cytoplasm of these cells is coagulated in the dogs that live for twenty-four hours or longer following the inoculation. Colloid or hyaline droplets are present in some of the tubular epithelial cells (Fig. B, 1). The walls of these tubules appear normal, although the injury to the epithelial cells may be very extensive.

The lumina of tubules are often filled with albumin, cellular debris, and hyaline casts. Some of the latter are calcified.

*Staphylococcus toxin injected intrarenally.* The renal lesions in this group of dogs are somewhat different from those which follow the intravenous injection

of staphylococcus toxin. The kidney on the side in which the renal artery is injected is the only one to vary from the normal. The gross appearance of these kidneys varies. The lesion is influenced by the length of time elapsing between the injection of the toxin and death. The injected kidneys from the dogs that died during the first four days of the experiment are swollen and hemorrhagic. Frequently the cortex shows focal hemorrhagic areas which are separated by apparently normal renal tissue. The kidneys from the dogs which survive for four days or longer vary widely in their gross appearance. Some are normal in size while others are atrophic (Fig B 7). The cortex may be irregular and scarred. Petechiae are present in the renal cortex of one of these dogs that lived for sixteen days following the intrarenal inoculation of the toxin (Fig B, 5).

The vascular lesions in the kidneys are very conspicuous in this group of dogs. Thrombi are present in branches of the renal artery proximal to the kidney in 3 of the animals. A majority of the other 9 dogs also show changes in the medium and small renal arteries. The walls of some of the arteries are infiltrated with polymorphonuclear leucocytes in the animals that survive the effects of the toxin for the shortest periods. Fresh old and organizing thrombi are present in other renal vessels. Necrosis in the cortex resembles that resulting from vascular obstruction. The renal tissue between these areas of necrosis shows an atrophy of some of the tubules and dilatation of others. The lumina of the latter are frequently filled with albumin red blood cells, and hyaline casts. The epithelial cells lining these tubules are usually smaller than the normal and stain more deeply with the basophilic stains. Some of the glomeruli are partially or completely fibrosed others are represented by a few greatly dilated capillaries (Fig A 3). Red blood cells sometimes fill Bowman's space and extend down into the convolution portion of the tubules. The interstitial tissue frequently has proliferated.

No pathologic changes are present in the kidneys of one of the two dogs in the control experiment. The dog that died had a wound infection and focal abscesses in both kidneys.

#### DISCUSSION

The present investigation shows that staphylococcus toxin when injected either intravenously or intrarenally injures the endothelial cells of the blood vessels, glomeruli, and the epithelium of the renal tubules. Thrombosis and infarction often result from injury to the endothelium of the blood vessels.

The early renal lesions following the intravenous injection of staphylococcus toxin are found primarily in the endothelium of the capillaries, in the glomeruli, and in the epithelial cells of the tubules. Staphylococcus toxin produces the same type of injury when given intravenously as it does when it is given intrarenally. However, when inoculated by the latter route, lesions resulting from the vascular change frequently completely overshadow those produced by the toxin in the glomeruli and tubules.

The effect of the procedure used in the intrarenal arterial injection may have had some influence on the development of the lesion. Whether or not

the toxin was in the same concentration when it reached the kidney following the intravenous injection as it was after the intrarenal inoculation is not known. It is interesting, however, to note that the severity of the process apparently was influenced by the amount of toxin injected. The variation in susceptibility of the different dogs was marked.

The tubular epithelium and the capillary endothelium show all the changes from cloudy swelling to complete destruction. Sometimes the renal lesions following the intravenous injection of staphylococcus toxin are so diffuse that death occurs very early. It is difficult to produce renal injury by the intravenous injection of the toxin without the process being so diffuse and severe that the animal dies acutely. The lesions produced by the toxin following the intrarenal injection are complicated by the infarcts which develop secondarily to the vascular injury.

The renal lesions which result from occlusions in the vessels can usually be differentiated from those resulting from the action of staphylococcus toxin. Those changes produced by the former involve both the parenchymatous tissue and the stroma, while the latter affect primarily only the parenchymatous tissues.

The glomerular lesions observed in this study resemble those produced by Ahlstrom in the rabbit by injecting intravenously staphylococcus toxin in combination with horse serum.<sup>6</sup> The renal lesions he thought were of allergic origin because they differed distinctly from those he observed in the rabbit inoculated with only staphylococcus toxin. Since no foreign serum was given to the dogs in this experiment, and, furthermore, since only one injection of toxin was given to many of the animals, it would appear most unlikely that the pathologic changes are the result of any allergic manifestation.

The association of glomerular and tubular lesions with an elevation in the nonprotein nitrogen indicates renal dysfunction. Some of the lesions in the dog following the intravenous and intrarenal injection of staphylococcus toxin are very similar to some of the lesions previously described in the human kidney in cases of staphylococcus infection.<sup>7</sup>

#### SUMMARY

Staphylococcus toxin injected intravenously and intrarenally in the dog injures directly the endothelium of the small renal arteries, glomerular capillaries, and the capillary plexus around the renal tubules, and also the tubular epithelial cells. Obstruction in blood vessels may occur secondarily to the vascular injury.

The renal lesion is accompanied by a retention of nonprotein nitrogen in the blood.

The histologic changes are frequently similar to those lesions observed in the human being in cases of glomerular nephritis.

#### REFERENCES

1. Neisser, M., and Levaditi, C.: *Action de la toxine staphylococcique sur le rein*, Cong. internat. Nied., Paris 13: 475, 1900.
2. Rigdon, R. H., Joyner, A. L., and Ricketts, E. T.: *A Study of the Action of a Filtrate of Staphylococcal Toxin on the Kidneys of Normal Rabbits*, *Am. J. Path.* 10: 425, 1934.

- 3 Von Glahn, W C, and Weld, Julia T The Effect of Staphylococcus Aureus Toxin on the Kidney, *J Exper Med* 61 1, 1935
- 4 Glynn, J H The Pathogenesis of Cortical Necrosis of the Kidneys in Rabbits Following the Injection of Staphylococcus Toxin *Am J Path* 13 593 1937
- 5 Rigdon, R H Inhibitory Action of Sodium Chloride on Skin Necrosis Produced by Staphylococcus Toxin, *Arch Path* 22 763, 1936
- 6 Ahlstrom, Carl G Zur Pathogenese der akuten diffusen Glomerulonephritis, *Acta path et microbiol Scandinar Suppl* 29 pp 1 174 1936
- 7 Rigdon, R H Renal Lesions in Staphylococcus Aureus Infections and Their Relation to Acute Glomerular Nephritis, *Arch Int Med* 57 117 1936

## ALIMENTARY LIPEMIA IN YOUNG DIABETICS WITH EVIDENCE OF LIVER DAMAGE OR DYSFUNCTION\*

WITH A NOTE ON THE EFFECT OF BETANE ADMINISTRATION ON LIVER FUNCTION  
AND ON ALIMENTARY LIPEMIA

ROBERT C LOWE, M D, NEW ORLEANS LA

AS THE result of the work of Hartley,<sup>1</sup> Leathes and Meyer Wedell,<sup>2</sup> London,<sup>3</sup> Artom,<sup>4</sup> Best,<sup>5</sup> Aylward, Channon, and Wilkinson and their associates, to mention only a few, the liver has come to be recognized as playing a definite role in the normal metabolism of fats. On the other hand the exact changes which fats undergo in the normal liver have not yet been definitely worked out. As the work on these problems has progressed attempts have been made to measure disturbances of fat metabolism in the diseased liver by comparing the degree of lipemia produced by the oral or the intravenous administration of fats with the lipemia observed in normal persons under the same test conditions.<sup>7-10</sup>

Nachlas and his associates,<sup>9</sup> studying the lipemic curves induced by intravenous injection of a fat emulsion in dogs before and after the production of experimental liver damage, interpreted their results as showing that the liver plays a part in the removal of fat from the blood, and that damage to this function is reflected in the form of the blood fat curves as an increase in the degree of lipemia. They point out, however, that the curves tend to return to normal form in spite of persistent anatomic liver damage. Sullivan and Feishtand,<sup>8</sup> who studied fat absorption curves obtained from patients with various types of liver disease, concluded that these curves were of value as an index of liver function. This conclusion was based on the finding that such patients exhibited an abnormally prolonged elevation of the blood fat level, though the elevation itself did not exceed the normal degree.

Attention was soon directed to fat metabolism in diabetes by the work of Bloor,<sup>11</sup> Man and Peters,<sup>12</sup> Blay,<sup>13</sup> and others, and studies on alimentary lipemia in such subjects have been carried out by several workers.<sup>8-13-14</sup> Blay was unable to demonstrate any abnormal variations of lipemia (p fraction, including neutral

\*From the Department of Internal Medicine and the Pathological Chemistry Laboratories University of Iowa Hospitals Iowa City and the Department of Medicine School of Medicine Louisiana State University New Orleans

Received for publication December 17 1938

fat and free cholesterol) in a series of diabetics. He concluded, therefore, that there was no relationship between the severity or the degree of control of the diabetes and the form of the blood fat curve. Ilejda,<sup>14</sup> who studied the lipemia curves in a group of nondiabetics, found that the ratio of the highest blood fat level to the fasting level varied from 1.27 to 1.35, which he considered to be the normal range. In another group, consisting of 3 diabetics, 1 patient with obesity, 1 with adiposogenital dystrophy, and 1 with myelogenous leucemia, he found ratios ranging from 1.4 to 1.58. He assumed on the basis of these higher ratios that these patients had a decreased tolerance for fat.

Sullivan and Fershtand included in their study of alimentary lipemia in liver disease a group of diabetic subjects, some of whom exhibited a delayed fall of their lipemia. They do not make it clear, however, whether they assume that the diabetics who showed a prolonged elevation of the blood fat curve had liver damage. Hanssen<sup>15</sup> reported a series of diabetics with enlargement of the liver in none of whom liver function tests (the urobilinuria test of Schlesinger and the choleic acid test of Hays) showed any variation from the normal.

#### MATERIALS AND METHODS

This contribution has two purposes: (1) to report a study of the fat absorption curves in 10 young diabetics who showed definite evidence of liver dysfunction or damage; and (2) to report a study of the influence of a lipotropic factor on these fat absorption curves and on liver function. There are no reports in the literature entirely comparable to our own, since other studies have been carried out on unselected groups of diabetics.

All of the diabetics studied were relatively refractory to control measures, and several showed symptoms of sympathetic imbalance similar to that observed by Man and Peters<sup>12</sup> in their contribution entitled *Serum Lipoids in Diabetic Acidosis*.

The presence of liver dysfunction or damage was determined by the existence of two or more of the following findings at the time the observations on alimentary lipemia were made:

- A. Elevated plasma bilirubin (Gibson and Goodrich).
- B. Decreased excretion of hippuric acid (Quick).
- C. Enlargement of the liver.
- D. Difficulty in controlling the diabetic state.

The fat absorption curves were obtained as follows:

The postabsorption level of the total plasma lipids was determined to be normal or at a constant level during a period of observation. After a fast of fourteen hours, a blood sample (oxalated) was withdrawn. The patient was then given a test meal consisting of one piece of toast, a cup of coffee without cream, and 1 gm. of butter per kg. of body weight. Venous blood samples (oxalated) were withdrawn one and one-half, three, four and one-half, and six hours after the meal.

The blood specimens were centrifuged at high speed for thirty minutes, and the total lipids were extracted from 5 c.c. of plasma by Bloor's method.

The whole extract was filtered off quantitatively and evaporated to dryness on the steam bath. The lipids were then extracted from the residue with petroleum ether, filtered quantitatively into a weighed dish, evaporated to dryness again, and kept overnight in a desiccator. The samples were weighed in the morning and the total lipids were calculated in milligrams per hundred cubic centimeters. Further fractionation of the lipids was carried out, but only the total lipid values are reported here with mention of the other fractions when the values seem especially important.

#### RESULTS AND DISCUSSION

The findings in our group of young diabetes are presented in Table I, arranged according to the first fasting blood lipid level and may be summarized as follows:

Four patients showed elevated plasma bilirubin (1.0 to 2.2 mg per cent). The norm is less than 0.5 mg per cent.

Six patients showed decreased excretion of hippuric acid (Quick). The norm is 3 gm excreted in four hours.

Seven patients showed livers enlarged 2 fingers or more below the costal margin.

All the subjects, it will be recalled, were young diabetes in whom it was difficult, if not impossible, to maintain adequate control of the blood sugar. It is thus evident that in each patient there are at least two findings which may be assumed to indicate gross anatomic changes or definite disturbance of the metabolic functions of the liver.

It will be noted that the postabsorption values of the total lipids fall within normal limits, except in Cases 9 and 10. In Case 10 hyperlipemia persisted over a period of nine months with very little variation although when the patient left the hospital his diabetes was well controlled. Such a "fixation" of the hyperlipemia has been described by Blum<sup>13</sup> who mentions three similar cases, one reported by Chauffard and two by Labbé. In Labbé's cases only the total cholesterol values were followed. Blum postulates a greatly damaged fat metabolism mechanism and a slow repair to explain the long continued hyperlipemia in these cases.

A comparison of the alimentary lipemia curves with other values will indicate how little correlation we observed between the clinical status of the patient's liver function and the shape of the blood lipid curve. If it be assumed that the degree of the elevation or the duration of the elevation of the blood lipids is an indication of the patient's tolerance for fats and if it be further assumed that liver damage decreases this tolerance and leads to abnormal elevations or prolonged elevations of the blood lipid curve, then liver function with reference to fat metabolism would not seem to be greatly altered in some of these patients. In some patients it might even appear that the feeding of fat stimulated its own metabolism in the body, corresponding to the "Bahnung" suggested by Blum.

In Table I there is given also the ratio employed by Heyda as a simple method of depicting the degree of lipemia. In our own cases this ranges from 0.69 to 1.52. Such a range indicates that all types of curves were encountered,



TABLE I

CASE NO.	AGE	DIET	PLASMA BILIRUBIN	LIVER ENLARGEMENT	HIPPURIC ACID EXCRETION (QUICK)	ALIMENTARY LIPEMIA*					CHANGE	CHANGE	HEJDA RATIO
						FAST-ING	1½	3	4½	6			
	Years	Gm. Per Day No. of Days	Mg. %		Gm.				Mg. %		Per cent	Mg.	
1	17	3 gm.—21 days	0.1	+++	2.94	444	553	556	675	548	+52	+231	1.52
2†	23	3 gm.—21 days	0.1	++	3.0	426	428	450	480	422			1.13
			0.1	+++	1.8	502	518	530	522	512	+5	+28	1.05
3	24	3 gm.—16 days	0.1	++	2.96	686	656	662	622	608			0.89
			2.2	0	1.6	612	560	676	626	608	+10	+64	1.1
			1.6	0	--	592	594	612	626	630			1.06
4	19	6 gm.—16 days	0.3	++	1.6	616	750	752	816	842	+37	+226	1.36
			0.2	+	2.64	950	908	1112	1060	958			1.17
5	16	6 gm.—12 days	0.2	++	2.0	692	758	916	1044	994	+51	+352	1.51
			0.2	+	2.92	848	884	956	872	858			1.13
6	23	6 gm.—21 days	1.8	0	3.1	706	620	653	536	486	-31	-220	0.69
			0.5	0	--	610	744	840	802	730			1.38
7	16	6 gm.—7 days	1.0	++	2.1	724	738	746	682	714	-6	-42	0.94
			0.5	+	3.0	760	882	872	814	872			1.17
8	18	6 gm.—7 days	0.3	+++	1.4	815	957	960	1022	1010	+25	+207	1.25
			0.2	+	3.0	912	906	946	962	852			1.05
9	22	6 gm.—7 days	0.2	0	1.03	1076	1034	1128	1128	1204	+12	+128	1.12
10	29	6 gm.—21 days	1.0	++	2.43	1348	1374	1288	1398	1428	+6	+80	1.06
			0.6	0	--	1392	1288	1328	1108	1330			0.79

\*The maximum fasting value in the control group was 883 mg. per cent, the minimum 179 mg. per cent, and the mean value 680 mg. per cent.  
†This patient was a dwarf.

TABLE II

CASE		ALIMENTARY LIPEMIA TOTAL PLASMA LIPID					CHANGE
	DATE	FASTING	1½	3	4½	6	
Mg %							Mg %
<i>Dog 1</i>							
1	11/29/37	752	656	674	606	606	-146
2	2/ 3/38	524	480	420	352	380	-172
3	12/13/37	372	452	448	428	454	+ 84
4	1/ 5/38	247	344	340	326	306	+133
<i>Dog 2</i>							
1	5/20/38	302	278	320	328	314	+ 26
2	5/24/38	248	268	294	282	274	+ 46
3	6/ 6/38	200	298	294	296	260	+ 98

TABLE III

FASTING TOTAL LIPID MG %	NO CASES	AVERAGE RISE OF LIPEMIA CURVES AT 3 6 HOURS	
		PER CENT	MG %
Data from Sullivan and Fershtand <sup>a</sup>			
400 500	8	85.0	313
500 600	16	57.0	289
600 700	12	31.7	198
700 1000	6	34.9	266
Data from Wechsler			
600 800	4	26.7	175
800 900	12	16.6	140
900 1100	4	8.5	80

varying from the paradoxical hypolipemia observed in diabetics by Blv to hyperlipemia comparable to that observed in the same type of subject by Blv, Heyda, and Sullivan and Fershtand

It was thought that certain data observed on depancreatized dogs might supply a possible clue to the explanation of these findings. Table II presents a series of alimentary lipemia curves obtained in 2 dogs which had been submitted to pancreatectomy several months before these studies were begun. During the period of observation the animals received a diet of two meals a day, each consisting of ground beef with about 8 per cent fat (300 to 400 gm) and sugar (25 gm). In addition, they were given bone ash (4 gm), cod liver oil, and vitamin B<sub>1</sub> once a day. During the period in which the tests were made each animal received 30 gm of a fatty acid mixture (1 part oleic to 2 parts palmitic) with 10 gm of glycerol daily to insure more adequate fat absorption. This precaution seemed necessary because, following pancreatectomy, the stools were extremely fatty, indicating a poor absorption of neutral fats. No pancreatic substance of any kind was administered. The blood lipid curves were obtained after a fasting period of twenty-four hours and a test meal of 100 cc of 40 per cent cream.

From the data obtained for both dogs it would seem that the form of the lipemic curve is in part, at least, a function of its initial level, whereas there appears to be an inverse relationship between the extent of the rise or fall of the curve and the initial level. In both dogs the curves vary from hypolipemic depressions through flat curves to hyperlipemic elevations.

Table III presents the figures obtained by calculation of the average rise of plasma fat in per cent and milligrams per 100 cc from the series of 43

normal patients reported by Sullivan and Fershtand<sup>8</sup> and from 20 curves on 17 normal subjects studied by Wechsler.<sup>7</sup> These figures suggest that in the normal individual there is a similar inverse relationship between the degree of lipemia and the postabsorption blood lipid level.

The data which we present in Table I show no semblance of correlation between the postabsorption values and the degree of lipemia. Although the relationship between liver dysfunction in human diabetics and fatty changes in the livers of depancreatized dogs is not definitely established, there seems no doubt that some relationship does exist. The outstanding differences seem to be that in the dog there is a marked drop in the total blood lipids and the plasma cholesterol esters. These findings, which were reported by Chaikoff and Kaplan,<sup>16</sup> were noted in our own experimental animals. It seems within the realm of possibility that diabetic subjects with normal or hypolipemic curves show postabsorption blood lipid levels which, although normal when compared with standards for normal subjects, may be, nevertheless, relatively hyperlipemic as regards the particular individual. Whatever pathologic physiology operates in the dog to lower the basal blood lipid level might very well be operating in these diabetics also, complicated by some factor tending to elevate the blood lipids above the basal level. The nature of this complicating factor is, however, not clear. Its operation in the depancreatized dog is indicated by the variations of the postabsorption blood lipid level (Table II). No adequate explanation is at hand, except, perhaps, that at the time the high postabsorption lipid values were obtained, the dogs required more insulin than when the values were low, which indicates a refractory state of the diabetes at these times.

#### THE EFFECT OF BETAINE CHLORIDE ON ALIMENTARY LIPEMIA

The work of Best<sup>5</sup> and his associates has shown that the normal metabolism of fat in the liver requires the presence of choline or a choline-like substance in the diet. In the absence of such a substance fat deposition occurs in the liver, and the percentage of neutral fats and cholesterol esters rises to abnormal levels. Best has found that betaine, like choline, will prevent this deposition. The curative effect of this therapy, however, seems less than the preventive.

In another phase of our study we attempted to determine the effect of betaine on hepatic function, as indicated by various liver function tests, and on the fat absorption curves. Nine of our 10 patients received from 3 to 6 gm. of betaine chloride daily over periods ranging from one to three weeks. At the end of these periods, the liver function tests and fat absorption curves were repeated. The data thus obtained are included in Table I and may be summarized as follows:

In 4 patients in whom the plasma bilirubin had been previously increased, there was a return to normal level in 3 patients and a slight decrease in the fourth patient. Rabinowitch<sup>17</sup> noted similar alterations.

In all patients on whom the Quick test was repeated there was a return to normal levels.

There was a variable, but not striking, degree of decrease in the size of the liver in the 7 patients in whom it was abnormally enlarged.

Several of the patients showed a definite improvement in their response to the measures instituted for the control of their diabetes. This improvement was particularly striking in 2 patients who had been in the hospital for several weeks prior to the beginning of this study and who had been very difficult to manage during that time.

The changes observed in the fat absorption curves are of interest, though it is difficult to evaluate the specific effect of the betaine because the reactions varied so definitely. There were two distinct types of reaction observed. In 4 of the 9 patients there was a definite rise (100 mg or more) of the postabsorption level of the blood lipids. In 4 patients there was no significant change. In the ninth patient there was a definite decrease of 96 mg. In Cases 1, 2, 4, 5, 8, and 10 there was a decrease in the degree of lipemia induced by the fat meal following betaine administration, as indicated by the Hcda ratio and in all except Case 2, in which there was a continuous decline of the blood lipids the lipid values returned to the fasting level at six hours in contrast to the original curves. It was in this group of patients that the greatest improvement in the diabetic state was observed.

In Cases 3, 6, and 7 the changes were exactly the reverse of those just mentioned. The original curves (obtained before betaine administration) either rose slightly or decreased below the fasting level. Those curves which rose above the fasting level returned to it at the sixth hour, whereas after the administration of betaine the lipemia curves of each of the patients showed an increased absolute and relative rise and a delayed return to the original level.

Other differences separate this second group of cases from the first group. Except for Case 10, which is an instance of 'fixed hyperlipemia,' the cases in this second group are the only ones which show an increased plasma bilirubin. They all show relatively high cholesterol ester percentages in the plasma, 70, 81, and 94 per cent, respectively, of the total cholesterol. A similar phenomenon was observed by Gardner and Gamsborough<sup>18</sup> in 4 cases of cholecystitis and cholelithiasis. Under the influence of betaine therapy the ester percentage in our own cases dropped by 15.7 per cent, 65.4 per cent, and 47 per cent, respectively, of their original percentage values. In this second group of patients, furthermore, no definite improvement was observed in their response to diabetic management.

The ester percentages in the first group were normal, and there was no significant alteration after the institution of betaine therapy. It seems likely, therefore, that some fundamental difference exists between these two groups of patients, but we do not have sufficient data available to make definite statements on this point.

#### SUMMARY

1 The alimentary lipemia was studied in a group of 10 young diabetics, all of whom gave evidence of liver dysfunction or anatomic liver damage. No correlation was found between the level or duration of elevation of the total blood lipids and the degree of liver damage indicated by several liver function tests.

2 Clinical and experimental evidence is presented which may serve to explain the difference in our results as compared with the reports in the litera-

ture, which suggest that in certain types of liver damage alimentary lipemia serves as an index of liver function.

3. A study was made of the effect of betaine chloride therapy on the liver function, diabetic status, and alimentary lipemia in this group of diabetic subjects. In all cases liver function was improved according to the tests employed. Two types of response were observed with respect to the alimentary lipemia, and the clinical control of the diabetic status seemed to differ according to the type of response. A decrease of alimentary lipemia was associated with an improvement in diabetic control, whereas an increase of alimentary lipemia was associated with no significant alteration.

4. A fundamental metabolic difference of some yet undefinable sort appears to exist which may account for the difference in response of these patients to both a "fat tolerance procedure" and betaine therapy for control of their diabetic state. The number of cases is too small, however, to warrant definite conclusions.

*Note:* After this paper was completed there came to our attention an article on this subject by Priscilla White and her associates (White, P., Marble, A., Bogan, I. K., and Smith, R. M.: Enlargement of the Liver in Diabetic Children. II. Effect of Raw Pancreas, Betaine Hydrochloride, and Protamine Insulin. *Arch. Int. Med.* 62: 751-764, 1938). Their data correspond with ours in all respects, except that they observed a consistent decrease in cholesterol ester values from the normal of approximately 50 per cent, whereas we found values varying from 50 to 94 per cent.

I am grateful to Professor R. B. Gibson for his assistance and advice during this study, and to Dr. F. M. Smith for permitting me to make the necessary tests on the patients in his hospital wards.

#### REFERENCES

- Hartley, P.: On the Nature of the Fat Contained in the Liver, Kidney, and Heart, *J. Physiol.* 38: 353, 1909.
- Leathes, J. B., and Meyer-Wedell, L.: On the Desaturation of Fatty Acids in the Liver, *J. Physiol., Proc. Physiol. Soc.* 38: 33, 1909.
- London, cited by Bloor, W. R.: Annual Review of Biochemistry 3: 185, 1934.
- Artom, cited by Bloor, W. R.: Annual Review of Biochemistry 3: 185, 1934.
- Best, C. H.: The Role of the Liver in the Metabolism of Carbohydrate and Fat, *Lancet* 1: 1274, 1934.
- Aylward, F. X., Channon, H. J., and Wilkinson, H.: The Liver and Fat Metabolism, *Biochem. J.* 29: 169, 1935.
- Wechsler, H. F.: Variations in the Total Blood Lipids in Alimentary Lipemia, *Arch. Int. Med.* 50: 37, 1932.
- Sullivan, M., and Fershtand, J. A. B.: Fat Absorption, Its Value as an Index of Function of Liver, *Arch. Int. Med.* 55: 834, 1935.
- Nachlas, A., Duff, G. L., Tidwell, H. C., and Holt, L. E.: Liver Function as Tested by the Lipemic Curves After Intravenous Fat Administration, *J. Clin. Investigation* 15: 143, 1936.
- Feigl, J.: *Biochem. Ztschr.* 88: 53, 1918.
- Bloor, W. R.: Fat Assimilation, *J. Biol. Chem.* 23: 317, 1915; 24: 447, 1916.
- Man, E. B., and Peters, J. P.: Serum Lipoids in Diabetic Acidosis, *J. Clin. Investigation* 13: 237, 1934.
- Blix, G.: Studies on Diabetic Lipemia. Part I. *Acta med. Scandinav.* 64: 142, 1926; Part II. *Acta med. Scandinav.* 64: 175, 1926; Part III. *Acta med. Scandinav.* 64: 234, 1926.
- Hejda, B.: Alimentary Lipemia, Study of Lipemic Curve, *Am. J. M. Sc.* 180: 84, 1930.
- Hanssen, P.: Enlargement of the Liver in Diabetes Mellitus, *J. A. M. A.* 106: 914, 1936.
- Chaikoff, I. L., and Kaplan, A.: The Blood Lipids in Completely Depancreatized Dogs Maintained With Insulin, *J. Biol. Chem.* 106: 267, 1936.
- Rabinovitch, I. M.: Effects of Betaine Upon Cholesterol and Bilirubin Contents of Blood Plasma in Diabetes Mellitus, *Canad. M. A. J.* 34: 637, 1936.
- Gardner, J. A., and Gainsborough, H.: Blood Cholesterol Studies in Biliary and Hepatic Disease, *Quart. J. Med.* 23: 465, 1930.

## THE EFFECT OF INTRAVENOUS INJECTIONS OF MAGNESIUM SULFATE ON THE VOLUME OF THE EXTREMITIES\*

VICTOR G. HAURY, A B, M D, PHILADELPHIA, PA.

IT HAS been shown<sup>1</sup> that intravenous injections of magnesium sulfate into experimental animals produce a dilation of the capillaries and an increase in the volume of the splanchnic organs. The present investigation was undertaken to determine if magnesium would produce a similar vasodilation and an increase in the volume of the extremities, when injected into the human subject.

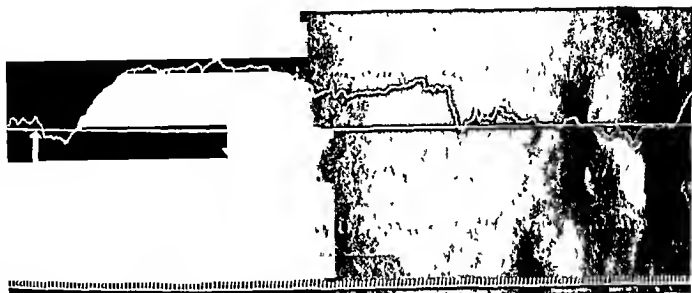


Fig. 1.—Male, E S, aged 40 years. Plethysmographic record of right foot. Twenty cubic centimeters of 10 per cent magnesium sulfate were injected in the interval between the arrows † †. Upstroke of the record indicates an increase in the size of the organ. Time in intervals of six seconds is shown in the bottom line.

**Method.**—Seven patients suffering from vasomotor disorders were chosen as the subjects for this investigation. Each patient was given 20 c.c. of 10 per cent magnesium sulfate at the rate of 4 c.c. per minute. The injections were made into the median basilic vein. Previous to the injection the arm or foot was enclosed in a glass oneometer. The oneometer was connected with a rubber tube to a modified Brodie bellows, which wrote its record on a smoked drum surface.

**Results.**—In every case a marked increase in the volume of the extremity was recorded following the intravenous injection of the magnesium sulfate. The dilation persisted for ten to thirty minutes following a single injection. The only subjective symptoms reported by the patients were a feeling of dryness and warmth of the mouth, and a generalized feeling of heat starting in the

\*From the Department of Pharmacology, Jefferson Medical College, Philadelphia.  
This investigation was made possible through a grant from Parke, Davis & Co. to Jefferson Medical College for research in science.

Received for publication, December 19, 1938

thorax and arms and progressing downward. Two patients became slightly drowsy. No respiratory depression was noted whatever. Ampoules of calcium gluconate were always kept available in case of emergency. One patient suffering from Raynaud's disease was given 2 c.c. of 50 per cent magnesium sulfate intramuscularly following the intravenous injection. This patient reported a relief of symptoms which recurred twenty-four hours later. Whether or not this relief was due to the magnesium therapy must be determined by further trials.

Fig. 1 shows a typical plethysmographic record of the lower right extremity following the injection of 20 c.c. of 10 per cent magnesium sulfate. The increase in the volume of the leg persisted for ten minutes in this case.

#### CONCLUSIONS

Intravenous injections of magnesium sulfate produce a marked increase in the volume of the extremities. By analogy with experiments on laboratory animals this increase in volume is believed to be due to an active vasodilation.

#### REFERENCE

1. Haury, V. G.: J. Pharmacol. & Exper. Therap. 65: 453, 1939.

---

### RAGWEED POLLEN: AN ARTEFACT IN TISSUE SECTIONS\*

EDWIN G. EBERTZ, B.S., AND WALTER C. LOBITZ, JR., B.S., CINCINNATI, OHIO

---

WITH the widespread distribution of ragweed pollen it is indeed curious that contamination of biologic specimens by these materials has not been reported often. We have had an interesting experience with ragweed pollen contamination of tissue prepared for microscopic examination.

A biopsy was taken of a skin lesion from a case of atypical scleredema in Cincinnati in the later summer of 1933. The section was placed immediately in formalin and sent to the pathologic laboratory. Detailed special staining was done and one set of slides, stained by Giemsa's method, showed occasional round spiculed bodies. These spiculed spheres, about six times the diameter of a red blood cell, had a thick shell-like structure surrounding a central darker staining area, either round, oval, or three-sided (uterus-like) in shape (Fig. 1). These bodies appeared to be within the tissue in some sections and on top of the tissue in others. There were no reacting cells present in relation to these spheres. There was no foreign body giant cell reaction present.

Curious as it may seem, a skin lesion (from the arm) during a recurrence of the same disease process in the same individual, in the fall of 1937, showed similar spheres. This biopsy was taken at the Cincinnati General Hospital and sent to the pathologic laboratory.

---

\*From the Department of Physiology, University of Cincinnati.  
Received for publication, December 21, 1938.

These spiculed bodies were not seen in the first set of biopsies taken in 1933. Because of the interest of the case itself, sections were sent to pathologists all over the world. During a detailed examination as regards search for bacteria, these bodies were found in certain sections of the biopsy taken in 1937. After this the first set of biopsies was re-examined and in a few of the sections these bodies were seen.

When these bodies were observed first there was considerable speculation. Many diagnoses were offered. Most of the opinions leaned toward some sort of "parasitic worm" or "ovum." It was obvious after the correct diagnosis had been made, that most of the workers in various fields were not familiar with ragweed pollen.



Fig. 1—Ragweed pollen granule (dried) lying to right of arrectores pilorum and to left of space artefact in the subcutaneous tissue of the skin lesion in an incidence of atypical scleredema. Note the absence of any reacting cells or foreign body giant cells.

Extensive investigation proved these bodies to be dried ragweed pollen granules (*Ambrosiaceae*) having no etiologic relationship to the disease entity, they were merely foreign bodies introduced during the process of fixing and staining.

The fixing, staining, and mounting of these sections in 1933 and 1937 occurred in different laboratories, each located in a different building. Both these buildings, however, are in close proximity to the same wind swept field, notorious for its ragweed. Since the workers in these and other laboratories in the same and bordering buildings use open windows in the late summer and fall months, it is strange that this contamination has not occurred or been recognized more frequently.



## SUMMARY

Suspicious microscopic bodies were seen in skin biopsies of the same disease in the same individual taken after an interval of four years. These bodies were found to be ragweed pollen and were merely contaminants occurring during tissue fixing and staining. The tissues were prepared in different laboratories of the same hospital in late summer of 1933 and fall of 1937. Adjacent to these laboratories was a huge wind-swept field filled with ragweed.

---

THE CULTURE OF HUMAN MARROW AS AN AID IN THE  
EVALUATION OF THERAPEUTIC AGENTS\*

---

STUDIES OF SULFANILAMIDE AND RELATED COMPOUNDS

---

EDWIN E. OSGOOD, M.D., PORTLAND, ORE.

---

THE chief methods now in use for the evaluation of therapeutic agents are animal experimentation and clinical investigation. Perfusion experiments and experiments on isolated fragments of tissue are also in common use. These methods are all of proved value, but all have certain limitations. It is the purpose of this article to point out some of the advantages which the methods developed for culture of human marrow offer in the study of therapeutic and noxious agents.

## METHODS

The vaccine vial technique has already been described.<sup>1</sup> It is very simple and well adapted for controlled quantitative studies. Briefly, it is as follows:

With a tight 10 c.c. syringe, aspirate from 1 to 10 c.c. of marrow.<sup>2</sup> Attach a 20 gauge needle and introduce the marrow into a 50 c.c. vaccine vial containing 25 c.c. of sterile citrated balanced salt solution. Centrifugate the vial containing the marrow, withdraw the supernatant liquid, mix the cells with the remaining liquid, and transfer to a volume index tube<sup>3</sup> capped with rubber dam. Centrifugate this, discard the supernatant fluid, withdraw all of the white cell layer and a little of the red blood cell layer, and transfer to a 30 c.c. Pyrex vaccine vial containing 8 c.c. of marrow culture medium. The medium consists of 35 per cent human cord serum and 65 per cent balanced salt solution. Mix well. This procedure concentrates the nucleated cells and eliminates most of the akaryocytes (nonnucleated erythrocytes) which have a tendency to inhibit growth. Mix thoroughly, withdraw 0.5 c.c., and do a total nucleated cell count according to the usual technique for white cell counts. Dilute the remaining marrow in the vial with the required amount of marrow culture medium to give a total nucleated cell count of 1,000 to 2,000 per c.mm. Mix thoroughly, and sub-

---

\*From the Department of Medicine and the Division of Experimental Medicine, University of Oregon Medical School, Portland.

Read before the Section on Pharmacology and Therapeutics of the American Medical Association, San Francisco, June 16, 1938.

Received for publication, December 28, 1938.

divide into a series of 30 e e vacuine vials placing 8 (5 to 15) e e in each vial. Leave one for a control, and to the others add the desired quantity of a sterile solution in balanced salt solution of any substance to be tested. Place in the incubator. Change the medium by centrifugation and withdrawal of the supernatant fluid every forty eight hours. Change the gas mixture once a week. Samples of the mixed cultures may be withdrawn at any time for quantitative chemical, hematologic, bacteriologic or serologic study.

The improved apparatus for large scale study<sup>4</sup> has been described in detail. It offers the additional advantages of maintaining under constant control the oxygen and carbon dioxide tensions, the composition of the medium, its rate of aeration, and the elimination of waste products. In other words it provides for the functions of a lung, aeration and kidney. For most purposes, however, the simpler vacuine vial method will prove satisfactory.

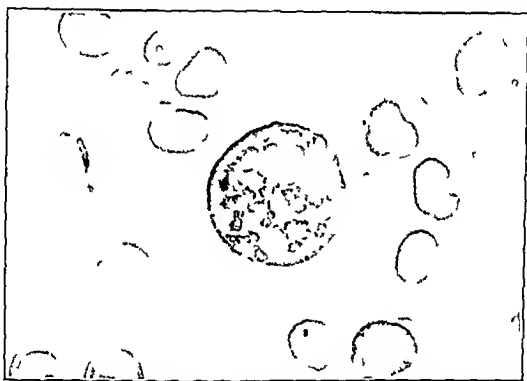


Fig 1.—Karyoblast (megakaryoblast) arrested in the metaphase of mitosis from a sixteen hour old culture of human marrow containing 1:250,000 colchicine. Wright's stain  $\times 1800$ .

*Advantages of These Methods*—Human marrow is readily available from either sick or well persons by the simple and relatively painless sternal puncture method.<sup>2</sup>

It seems likely that conclusions based on the study of human cells should be more directly applicable to human disease than studies on animals or on animal tissue.

These cells are separate and countable so that the number of each type of cell present at any time may be calculated, including the number of cells in process of mitotic and amitotic division. No other method of tissue culture permits this accurate determination of the amount and type of tissue investigated. With the aid of colchicine,<sup>2</sup> which arrests mitoses in the metaphase, it may be possible to determine the number of mitoses in any desired period of time (Fig 1).

The morphology of the cells is much better preserved than in fixed tissue sections.

The volume is sufficient so that any of the quantitative methods of hematology, chemistry, bacteriology, or serology which may be done on blood may be done on these cultures. We have already performed the following quantitative procedures on such cultures: erythrocyte count, hemoglobin estimation, total nucleated cell count, differential cell count, reticulocyte count peroxidase stain, studies of cell motility in supravital preparations, quantitative studies of phagocytosis, counts of mitoses with and without colchicine, quantitative determinations of dextrose, urea nitrogen, sulfanilamide, pour plate colony counts, and comparisons of different types and concentrations of antisera. The quantitative procedures used are those<sup>6</sup> with which any well-trained technician is familiar.

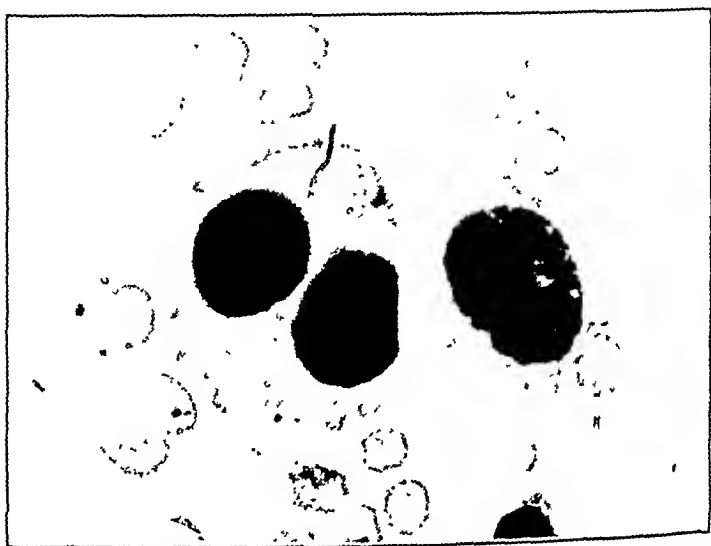


Fig. 2.—Tumor cells from a two-day-old culture of ascitic fluid from a patient with a very anaplastic carcinoma of the stomach. Wright's stain  $\times 1,500$ .

The vaccine vial technique is extremely simple, requiring no apparatus not present in a well-equipped laboratory. Most of the manipulations are done with syringe and needle through vaccine vial caps sterilized with 70 per cent alcohol, so that undesired bacterial contaminations are rare.

A type of control is possible which is not attainable in animal experimentation or clinical investigation, since the culture may be thoroughly mixed in one vial after adding the noxious agent under investigation, and then equal volumes transferred to each of 5 to 8 vials. One of these is left as a control, and to the others, varying concentrations of the therapeutic agent under investigation may be added. One can be certain then that there are actually in each vial the same number of each type of cell, the same quantity of identical medium, and the same concentration of the noxious agent, something which one never can be sure of in animal experimentation or clinical investigation.

The variables of absorption and excretion, inherent in animal experimentation and clinical investigation, are not present, so that by measuring the amount of the therapeutic agent introduced and making a quantitative determination of it at the end of the experiment, one can be certain of the actual level maintained.

A measured, thoroughly representative sample may be removed at any desired interval for quantitative study without harming the remainder of the culture. Knowing the total volume of culture from the study of this sample, the quantitative characteristics of this culture may be determined. This is not possible with any other method of studying living vertebrate tissue.

By teasing the cells apart in the fluid medium, many tissues from animals and man can undoubtedly be grown by these methods. We have already cultured human marrow from healthy persons and from patients with many diseases, normal<sup>7</sup> and leucemic blood, lymph nodes, splenic tissue, and cells from an anaplastic carcinoma of the stomach (Fig 2)

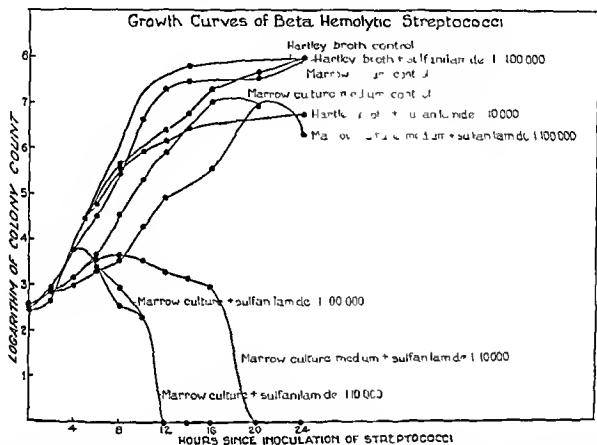


Fig 3.—Logarithmic growth curves from marrow culture, marrow culture medium, and Hartley broth. These media in 3 vaccine vials were inoculated with equal numbers of beta hemolytic streptococci, mixed, and each divided equally into 3 vials, one being the control, and the other two containing enough sulfanilamide to give concentrations of 1:10,000 and 1:100,000. Samples were removed at intervals, and pour plate colony counts were made from suitable dilutions. In this and subsequent curves, 1 equals 10 colonies per c.c., 2 equals 100 colonies per c.c., etc., up to 8 equals 100,000,000 colonies per c.c.

Life is readily demonstrable by motility in supravital preparations or by phagocytosis a few minutes after addition of a vaccine

The medium contains nothing not present in similar concentration in the fluid surrounding cells in the human body, and has been demonstrated<sup>8</sup> to be superior to Tyrode's or Ringer's solution for permitting cell division, maturation, and functional activity.

*Limitations of These Methods.*—As yet it has not been possible to obtain unlimited quantities from a single inoculation. Structural relationships are not preserved. The method is not suitable for the culture of whole organs. The interaction of the various organs and tissues on each other cannot be duplicated as in the intact animal. Substances which prove nontoxic for the cells investigated may be toxic for other types of body cells.

With the techniques at present in use, the cultures show deterioration after one to three weeks, although living cells have been found at fifty days. The method must, therefore, be further perfected before experiments lasting more than one or two weeks can be given quantitative interpretation. The extent to which results obtained in marrow cultures will be duplicated in the body remains to be determined, although results so far obtained appear to show close correlation. It is unlikely that the medium contains everything present in normal tissue in the same concentrations. The cord serum or marrow from different sources may vary so that conclusions should be drawn only from experiments in which the controls have actually been mixed in the same vial with the cultures with which they are compared.

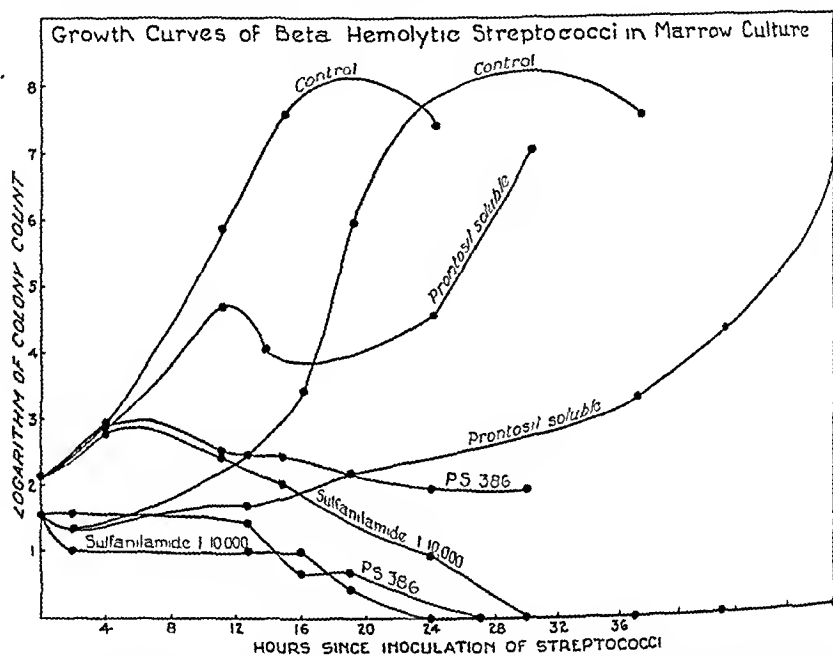


Fig. 4—Logarithmic growth curves of experiments 168 and 169. These curves were plotted from pour plate colony counts made at the indicated intervals. In each experiment the cultures were mixed in 1 vial after inoculation with beta hemolytic streptococci, and then equal volumes were placed in each of 4 vials, 1 of which was left as a control; to another, 1:10,000 sulfanilamide, that is, 10 mg. per 100 c.c., was added; to a third, prontosil soluble in a concentration of 34 mg. per 100 c.c. was added; and to the fourth, P. S. 386 or prontosil maltoside was added in a concentration of 30 mg. per 100 c.c. The curves were calculated so that if all the theoretically possible amounts of sulfanilamide and prontosil maltoside the concentration of prontosil was 1:10,000.

The services of a full-time technician skilled in hematology and bacteriology are essential.

*Experiments Illustrating the Usefulness of the Methods.*—The growth curves in Fig. 3 are derived from the data in Table II from the report on the mode of action of sulfanilamide on the beta hemolytic streptococcus.<sup>9</sup> They illustrate the possibility of comparing controls with different concentrations of the therapeutic agent, in this case, sulfanilamide; of following quantitatively the effects on the noxious agent over a period of time; and of segregating the effects of cells from the effects of serum. To date no research or clinical data have been found which cannot be explained by the conclusions on the action of sulfanilamide derived from this study.

The growth curves in Figs 4 and 5 have not heretofore been published. They illustrate the possibility of quantitative comparison of different therapeutic agents on the same noxious agent. In animal experimentation directed to the solution of the same problem, errors might be due to failure of the particular species to release sulfanilamide from the more complex compounds. This release occurs in the human body and in these cultures. Errors might be due to giving only 1 to 3 doses a day, which would give an intermittent high concentration instead of the uniform low concentration readily proved to be necessary by the marrow culture method. Therefore a slowly absorbed or excreted compound might appear superior to a more effective compound given at too infrequent intervals to maintain a uniform concentration.

TABLE I

COMPARISON OF THE EFFECTS OF TYPE I ANTIPNEUMOCOCCUS RABBIT SERUM AND 1:10,000 SULFANILAMIDE WITH THE SAME DOSE OF ANTISERUM ON TYPE I PNEUMOCOCCUS INFECTIONS IN MARROW CULTURES\*

HOURS	0	18	24	48	70
Antiserum, 2.5 units per c.c.	6	2,500	70,000	175,000	100,000
Antiserum, 2.5 units per c.c. plus sulfanilamide	6	10	1	15	4,000
Antiserum, 12.5 units per c.c.	6	60	1,300	35,000	15,000
Antiserum, 12.5 units per c.c. plus sulfanilamide	6	20	0	0	0

\*The control cultures and the cultures containing 1:10,000 sulfanilamide alone uniformly reached counts of 10,000,000 to over 100,000,000 per c.c. within twenty-four to thirty-six hours; the controls reaching these levels six to twelve hours before the cultures containing sulfanilamide.

Quantitative studies on the comparative action of sulfanilamide and antipneumococcus serum on the type I pneumococcus<sup>10</sup> from which Table I is taken, illustrate the possibility of studying the interaction of varying concentrations of two different therapeutic agents on the same noxious agent. Similar studies on the type II pneumococcus, not heretofore reported, are given in the growth curves in Fig 6. Note how definitely this shows the superiority of combinations of sulfanilamide and antipneumococcus serum over the same concentrations of either alone. To obtain this information by clinical investigation, it would have been necessary to study hundreds of cases of the same type of pneumococcus pneumonia during one season, with treatment begun on the same day of the disease, and identical except for the use of specific antiserum or sulfanilamide. These would have to be divided into at least four unselected groups for therapy: controls, those given sulfanilamide alone, those given antipneumococcus serum alone, and those given both sulfanilamide and antipneumococcus serum. Even then there would be many variables of size of the infecting dose, virulence of the organism, race, age, sex, heredity, alcoholism, syphilis, etc., not adequately controlled as in the marrow culture experiments. Furthermore, the controls and those treated with sulfanilamide alone would probably have had a higher mortality than those treated with adequate amounts of antiserum as at present, so that some lives would have been lost unnecessarily. One would even then not know if smaller amounts of antiserum might justifiably be tried with sulfanilamide. It will still be necessary to study a large series of patients with

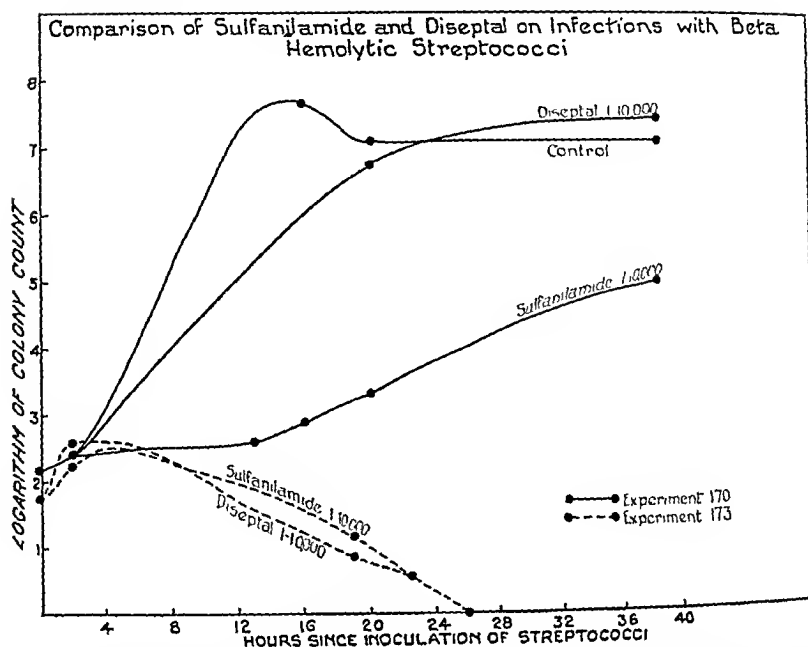


Fig. 5.—These logarithmic growth curves were derived from experiments performed in the same manner as those in Fig. 4. The actual concentration of disseptal was 10.3 mg. per 100 c.c. Although disseptal is effective, it is apparently not as effective as sulfanilamide, and the manufacturers have withdrawn it from clinical use because of its tendency to produce polyneuritis.

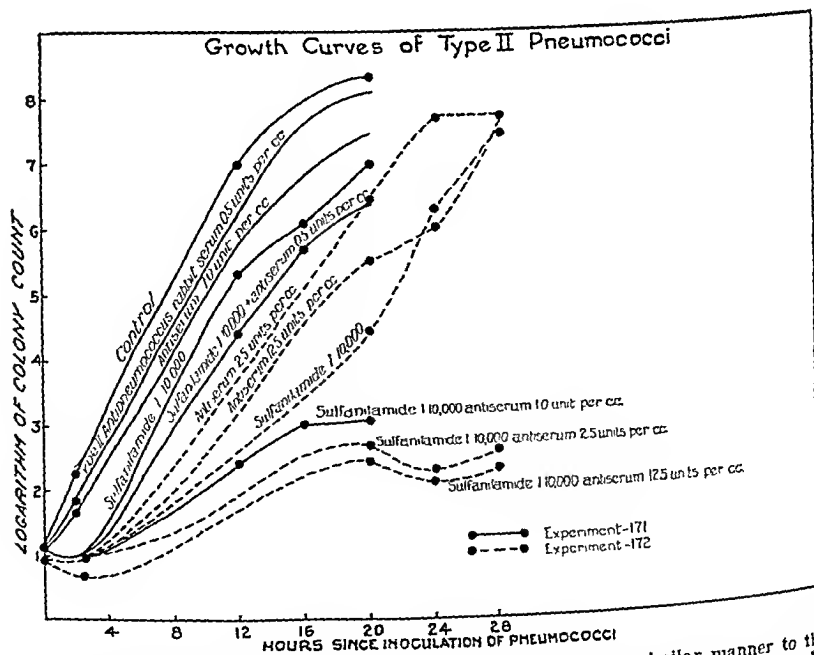


Fig. 6.—These logarithmic growth curves were obtained in a similar manner to those for the type I pneumococcus. Sulfanilamide plus antiserum is far more effective than the corresponding concentrations of either alone. The chief differences from the type I pneumococcus experiments are that type II pneumococci multiply more rapidly and reach a higher count in the controls; sulfanilamide alone is slightly more effective than against type I; and type-specific antiserum is slightly less effective than in the type I experiments.

pneumonia, alternating antiserum alone with antiserum plus sulfanilamide. With this work, however, as a guide definite information ought to be derived much more quickly. Such studies are already in progress<sup>11</sup> and at present appear to be corroborating our conclusion that for the effective action of sulfanilamide, specific bacteriostats must be present be introduced, or be developed.

#### PROBLEMS WHICH SHOULD BE INVESTIGATED BY THESE METHODS

Problems in many fields of medicine offer promise of solution by these marrow culture methods. Only actual trial will determine whether any of them may be solved. A few are here listed as examples of the type of investigation to which we feel this method is suited. Many others will occur to anyone who studies the possibilities of the methods.

**Metabolism** Studies should be made of the effects of variation in oxygen or carbon dioxide tension, temperature and of excess or deficiency of each hormone, vitamin, mineral, amino acid, etc.

**Hematology** Studies should be made of the histogenesis of the cells,<sup>12</sup> the growth characteristics of marrow from each disease, the effects of therapeutic agents on marrow from each disease, the effects of serum from patients with blood dyscrasias on normal marrow, etc.

**Malignancy** The effects of carcinogenic agents on normal cells, and of alpha, beta, and gamma rays, arsenic, etc., on leukemic or malignant cells should be investigated.

**Therapeutics** Studies should be made of the effects of sulfanilamide and related compounds on every known microorganism, of the effects of antipernicious anemia principle on pernicious anemia marrow, of iron on the marrow of hypochromic microcytic anemia, and of the effects of toxins such as lead, benzol, aminopyrine, etc.

**Bacteriology** The course of infections with each known microorganism, and the influence on this course of drugs and biologic and physical agents should be investigated. Attempts should be made to grow *Treponema pallidum*, viruses, and other organisms difficult to grow outside the human body.

**Immunology** Antibody production, allergy, the effects of antisera, anti-toxins, and bacteriostatic or antiseptic agents on infections with each known microorganism should be studied. Possibly, improved methods of standardizing biologicals could be developed.

**Parasitology** Attempts should be made to culture malaria, amoebae, trypanosomes, and other parasites difficult to grow outside the human body, and studies made of the effects of therapeutic agents on these parasites.

#### CONCLUSIONS

These tissue culture methods should prove a valuable supplement to animal experimentation as a guide to clinical investigation, and should be of value in research in many fields of medicine and biology. Illustrative studies herein reported indicate that the action of prontosil soluble, disseptal, and prontosil maltoside could be explained by the amount of sulfanilamide released, and that



these compounds are less effective than equivalent amounts of sulfanilamide itself. These studies also indicate that in pneumococcic infections sulfanilamide plus type specific antipneumococcus serum should be more effective than either alone or than much larger doses of antipneumococcus serum alone.

We are indebted to the Winthrop Chemical Co., Inc., for supplying us with the prontosoluble, prontylin maltoside, and disceptal used in these experiments.

We also wish to thank Eli Lilly & Co. for kindly supplying us with the types I and II antipneumococcus sera used in this study.

#### REFERENCES

1. Osgood, E. E., and Brownlee, Inez E.: Culture of Human Marrow: Details of a Simple Method, *J. A. M. A.* 108: 1793, 1937.
2. Osgood, E. E., and Ashworth, Clarice M.: *Atlas of Hematology*, San Francisco, 1937, J. W. Stacey, Inc., pp. 205-206.
3. Osgood, E. E.: *A Textbook of Laboratory Diagnosis*, ed. 2, Philadelphia, 1935, P. Blakiston's Son & Co., p. 419.
4. Osgood, E. E.: Culture of Human Marrow: An Improved Apparatus for Large Scale Culture, *Am. J. M. Sc.* 195: 141, 1938.
5. Brues, A. M., and Cohen, A.: Effects of Colchicine and Related Substances on Cell Division, *Biochem. J.* 30: 1363, 1936.
6. Reference 3, pp. 324-458.
7. Osgood, E. E.: Length of Life of the Neutrophils, Eosinophils and Basophils of Normal Blood as Determined by Comparative Cultures of Blood and Sternal Marrow From Healthy Persons, *J. A. M. A.* 109: 933, 1937.
8. Gey, G. O., and Gey, Margaret K.: The Maintenance of Human Normal Cells and Tumor Cells in Continuous Culture. I. Preliminary Report: Cultivation of Mesoblastic Tumors and Normal Tissue and Notes on Methods of Cultivation, *Am. J. Cancer* 27: 45, 1936.
9. Osgood, E. E.: Culture of Human Marrow: Studies on the Mode of Action of Sulfanilamide, *J. A. M. A.* 110: 349, 1938.
- Osgood, E. E., and Brownlee, Inez E.: The Mode of Action of Sulfanilamide, *J. A. M. A.* 110: 1770, 1938.
10. Osgood, E. E.: Culture of Human Marrow: A Comparative Study of the Effects of Sulfanilamide and Antipneumococcus Serum on the Course of Experimental Pneumococcus Infections, *Arch. Int. Med.* 62: 181, 1938.
11. Kolmer, J. A.: Personal communication.
12. Osgood, E. E.: The Histogenesis, Classification and Identification of the Cells of the Blood and Marrow Based on Cultures and Hematologic Studies of Human Marrow and Blood, *Am. J. Clin. Path.* 8: 59, 1938.

# LABORATORY METHODS

---

## A SIMPLIFIED METHOD FOR CONTINUOUS INTRAVENOUS INJECTION INTO SMALL ANIMALS\*

C ARTHUR WOERNER PH D CHICAGO ILL

---

### INTRODUCTION

THIS method of continuous injection into small animals was developed in connection with a series of experiments to determine the effects of continuous intravenous injection of dextrose on the cytologic constituents of the cells of the islands of Langerhans (Woerner 1938). The guinea pig was used because of the extensive studies that have been made on the cytology of the islands of Langerhans in the pancreas of the guinea pig by R R Bensley. Continuous injection methods have been developed by Woodvatt (1920) and modified by Colwell (1930), Jacobs (1931) and Soslin (1933) which have been very successfully used on the dog for periods of many days. The method described in this paper has been used on the guinea pig for periods as long as twenty eight days.

The use of small animals has several advantages. In injecting a given quantity of a substance per unit of body weight much smaller quantities are used. As, for example, to inject 1 gm per kg of body weight in a 10 kg dog, 10 gm are needed, while to inject 1 gm per kg of body weight in a 500 gm guinea pig, 0.5 gm are used. With the method described in this paper it is possible to inject as many as three to five animals at the same time with one machine and, in addition, it requires much less space than is needed for dogs. In taking pieces of tissue for histologic and cytologic studies, a piece of a given size in the guinea pig represents a larger part of an organ than would be the case when using a larger animal. Since methods of blood analysis are available, requiring samples as small as 0.1 cc, many experiments can be done which formerly required much larger samples. In the experiments on the continuous injection of dextrose, blood sugar determinations were made using 0.1 cc samples (Miller and Van Slyke, 1936). Basal metabolic rates can also be determined (Cole and Womack, 1934).

*Preparation of Apparatus*—The continuous injection machine is driven by a synchronous electric motor and can be made to hold as many as 5 syringes (Fig 1). The machine was so made that, with the dog c moving one tooth of the ratchet gear d per revolution and the shaft of the motor revolving 2 r p m, a 50 cc syringe injects 1 cc per hour. The eccentric b is mounted so that it

\*From the Department of Anatomy the University of Chicago  
Received for publication July 7 1938

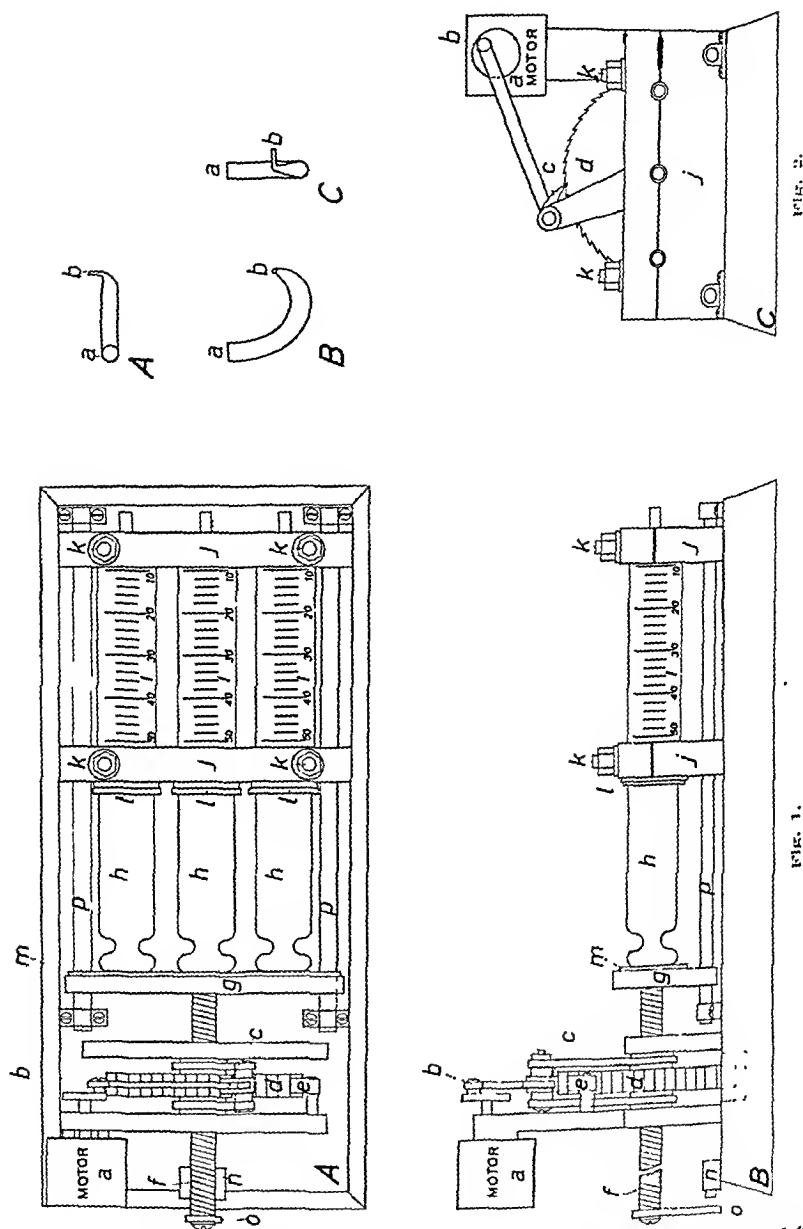


FIG. 1.

Fig. 1.—The continuous injection apparatus. A, Viewed from above. B, Viewed from the side. C, Viewed from the delivering end. a, Synchronous motor that drives the apparatus at a constant rate. The shaft of the motor rotates at 2 r.p.m. b, Eccentric on the shaft of the motor. The position of the eccentric can be changed so that the rate of injection can be changed. c, Dog activated by the eccentric (b). d, Ratchet gear activated by the dog (c). e, A second dog that keeps the ratchet gear (d) from reversing. f, Screw mounted in the center of the ratchet gear (d). g, Bar driven by the screw (f) when the ratchet gear (d) is rotated. h, Plunger of 50 c.c. syringe driven by the bar (g). i, 50 c.c. syringe. j, Syringe mounted in the wooden blocks that hold the 50 c.c. syringes in place. k, Bolts that hold the blocks (j) together. l, Rubber washers that protect the syringes from breakage. m, Thin rubber pad that protects the plungers of the syringes. n, Safety switch. o, Metal strip placed on the end of the screw (f) to contact the safety switch (n). The length of the screw is just enough so that the switch will turn off the motor and prevent breaking the syringes should one fail to reset the apparatus. p, Guide rods that keep the bar (g) aligned so that the plungers of the syringes are driven together.

Fig. 2.—The special glass tip. A, Viewed from above. B, Viewed from the side. C, Viewed from the front. a, The larger end of the tip that is connected to the rubber tubing leading from the continuous injection apparatus. b, The smaller end of the tip that has been drawn small enough to pass into the special rubber tubing. The tip has been bent in three dimensions so that it fits closely to the neck of the animal. The tip has been bent in three

can be adjusted to move the gear *d* 1 to 6 teeth per revolution and inject 1 to 6 c.c. per hour with each syringe. With the machine set to inject 2 c.c. per hour per syringe, different rates can be produced by connecting the tubing from more than one syringe together and inject 2, 4, or 6 c.c. per hour without the machine requiring attention more than once in twenty-four hours.

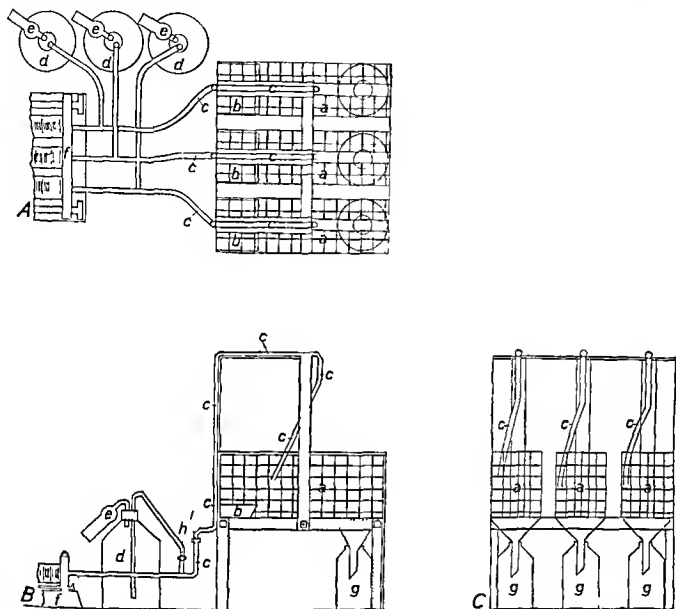
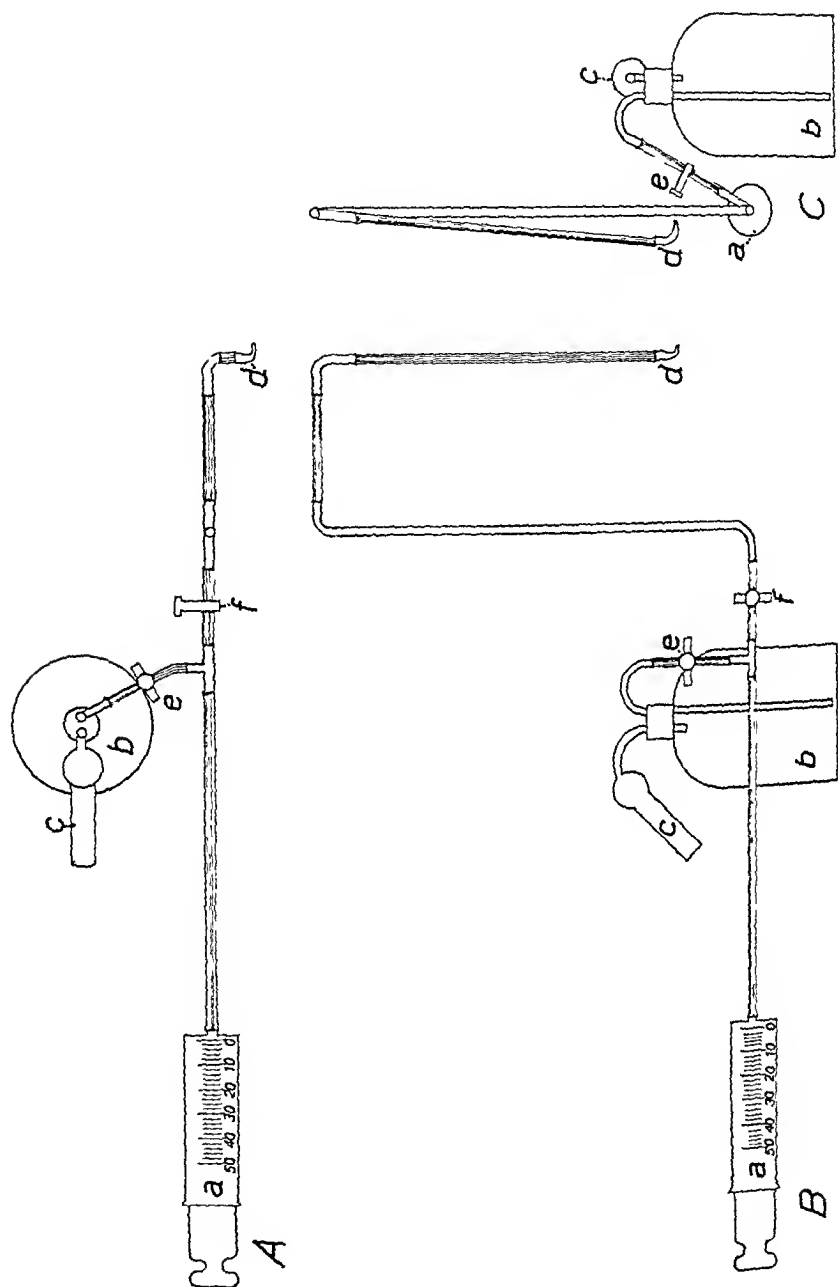


Fig. 3—The cages. *A*, Viewed from above. *B*, Viewed from side. *C*, Viewed from end. *a*, Cage in which guinea pig is confined. *b*, Metal tray in which food is placed. *c*, Glass and rubber tubing leading from injection apparatus to the animal. *d*, Bottle containing solution to be injected. *e*, Calcium chloride tube filled with cotton to prevent contamination of solution. *f*, The continuous injection apparatus. *g*, Bottle in which the urine is collected for analysis. *h*, Clamp on tube leading to solution bottle when machine is injecting. *i*, Clamp on tube leading to animal that is closed when syringes are being refilled from the bottle (*d*).

The glass tubing,  $\frac{1}{4}$  inch in diameter, is bent in the shapes required as indicated in the drawings of Figs 2 and 4. The specially bent glass tip, that connects the larger rubber tubing to the small intravenous rubber tubing, is drawn so that it is small enough to pass into the intravenous rubber tube and bent to fit the neck of the animal. If the injection of the desired substance is to be interrupted, a second bottle of saline is attached through a T-tube. The glass and rubber tubing and the syringe are assembled as shown in Fig. 4 and wrapped in a towel. They are then autoclaved at 15 pounds of steam pressure for fifteen minutes. If the solution to be injected is heat stable, it is put into the storage bottle and autoclaved at the same time.



Figs. 1.—Syringe and connecting tubing. A. Viewed from above. B. Viewed from side. C. Viewed from end. a, 50 cc. syringe. b, glass tube. c, clamp. d, glass tube filled with cotton to prevent con-  
 duction of solution. e, glass tube for connecting with intravenous rubber tubing. f, clamp put on tubing when refilling apparatus.

After the solution and apparatus have cooled, the plunger of the syringe is coated with sterile petrolennm jelly and the glass tube connected to the storage bottle (Fig. 3d and Fig. 4b). The system of tubing and the syringe are then filled with solution by manipulating the plunger of the syringe so that all of the air is expelled from the system. The syringe is then placed in the injection apparatus and where two or more animals are to be started at the same time this part of the apparatus is assembled for all of the animals before any of them are prepared for injection.

*Insertion of the Intravenous Tube into the Vein of the Animal*—The intravenous rubber tube (described by Jacobs 1933 and 1934) approximately 8 cm long, is put into the external jugular vein. This rubber tubing should be just small enough to pass easily through a No 16 needle. Smaller tubing has been found to be unsatisfactory. After shaving the neck of the animal the skin is washed with 70 per cent alcohol. The skin in the midline from the sternum

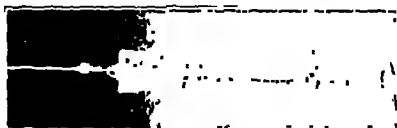


Fig 5—The 10 cc syringe with No 16 needle and intravenous tube (Jacobs 1934)

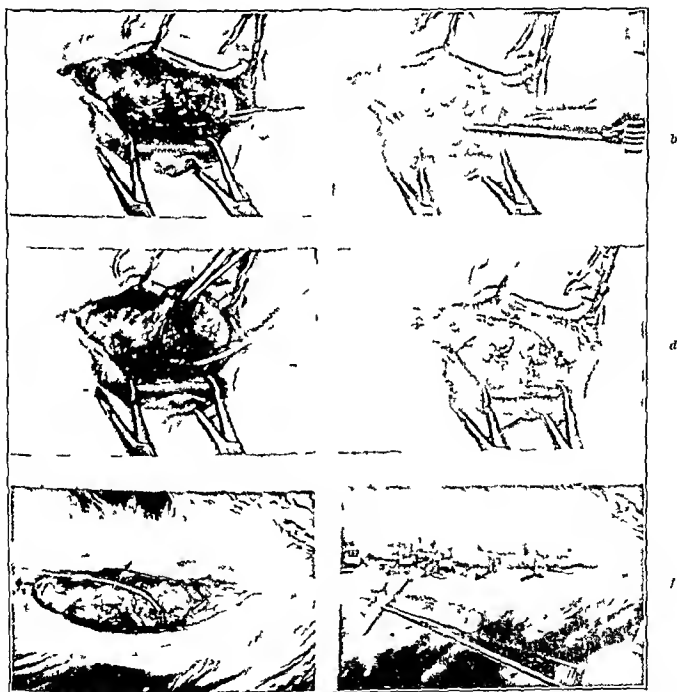


Fig 6—*a* Incision showing external jugular vein exposed with ligature under the vein. *b*, Incision showing insertion of needle into the vein. *c* Incision showing intravenous tube in the vein with needle and ligature removed. *d* Incision showing tube sutured to the fascia of the neck. *e*, Incision ready to be closed. *f* Incision closed with skin clips showing tube coming out between two of the skin clips.

cephalad for about 5 cm. is infiltrated with a 1 per cent solution of procaine hydrochloride. An incision is made extending from the sternum cephalad about 4 cm. The external jugular vein on one side is dissected clean and a linen thread is passed under it (Fig. 6a). A 10 c.c. Luer syringe, fitted with a No. 16 needle into which the intravenous rubber tubing has been previously inserted so that the intravenous tube extends almost to the point of the needle, is shown in Fig. 5. The tip of the needle is then put into the vein (Fig. 6b). Pressure is applied to the vein at the base of the neck, and a moderate traction is put on the ligature around the vein. The pressure on the vein at the base of the neck is then released, and the intravenous rubber tube is forced into the vein by gentle pressure on the plunger of the syringe. Care must be taken that not more than 2 or 3 cm. of the tube are put into the vein. A gauze pad is then placed over the vein to secure the tubing and the needle is withdrawn, leaving 2 or 3 cm. of the tube in the vein (Fig. 6c).



Fig. 7.—*a*, Neck of the animal with bandage, showing glass tip inserted into the intravenous tube. *b*, Glass tip ready to be taped to the neck of the animal. *c*, Animal ready to be put into the cage. The injection has already been started.

At this point a syringe fitted with a No. 20 needle is used to inject a small amount of saline into the vein by putting the No. 20 needle into the distal end of the intravenous rubber tube. Blood can now be drawn through the tube into the syringe if the tube has been properly inserted. The intravenous tube can now be stitched to the fascia of the neck of the animal in three different places (Fig. 6d), so that the tube forms a loop and the free end points caudad (Fig. 6e). The incision is then closed with skin clips and the tube is allowed to pass through the incision between two clips 1.5 to 2.5 cm. cephalad of the sternum (Fig. 6f). A gauze bandage, slit to permit the intravenous rubber tube to pass through it, is held in place by two tapes, one

at the base of the neck and one around the thorax. A small amount of saline is then injected through the tube and blood is drawn back into the syringe to test the patency of the tube. Another small quantity of saline should now be injected to clean out the tube.

The specially bent glass tip of the injection apparatus can now be put into the intravenous tube as shown in Fig 7a. The glass tip (Fig 7b) is taped securely to the neck of the animal (Fig 7c) and the animal placed in the cage (Fig 8).

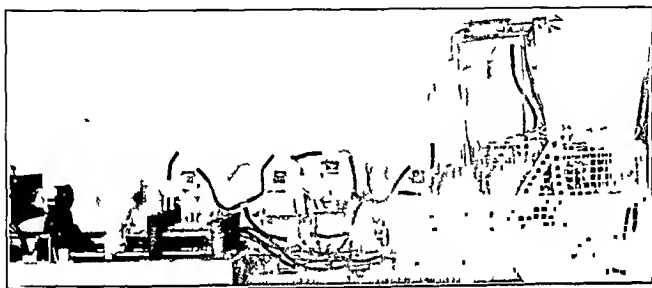


Fig 8—The complete apparatus in operation with three guinea pigs being injected. Each animal is receiving . . cc per hr.

In connecting the glass tip with the intravenous rubber tube (Fig 7a) it is necessary, of course, to avoid letting an air get into the system. This can be done if the injection machine is running. When more than one animal is to be started at the same time, the clamps Fig 3i and Fig 4f should be closed so that air in the tip being connected can be expelled and no solution need be injected into other animals already in the apparatus. In changing and refilling the apparatus care must be taken that the clamps are manipulated so that one does not draw blood from the animal back into the tube. When the syringes are being refilled, there should be a little pressure in the system. This can be produced by having both the clamps (Fig 3h and i, and Fig 4e and f) on the tubes and operating the apparatus by hand after which the clamp on the tube leading to the animal can be released.

The cages (Fig 3) are made of wire with a mesh of approximately  $\frac{1}{2}$  inch. They are just high enough so that the animal can stand comfortably, and wide enough for the animal to fit into, and yet small enough so that the animal cannot turn around or climb up the side of the cage. For guinea pigs weighing approximately 500 gm the cages are made about 8 cm wide, 10 cm high, and 30 cm long. A slit is left in the top of the cage to permit the tube to pass into the cage (Fig 3). A small metal dish (Fig 3b) is fitted into one end of the cage for food and a bottle (Fig 3g) with a funnel and filter paper is placed under the other end to collect urine for analysis.



## REFERENCES

1. Woerner, C. Arthur: Studies of the Islands of Langerhans After Continuous Intravenous Injection of Dextrose, *Anat. Rec.* 71: 33, 1938.
2. Bensley, R. R.: Studies on the Pancreas of the Guinea Pig, *Am. J. Anat.* 12: 297, 1911.
3. Bensley, R. R.: Studies and Relations of the Islets of Langerhans, The Harvey Lectures, Series X, Philadelphia, 1915, J. B. Lippincott Co., pp. 250-280.
4. Woodyatt, R. T.: An Improved Volumetric Pump for Continuous Intravenous Injection, *J. Biol. Chem.* 41: 315, 1920.
5. Colwell, A. R.: A Clock-Timed Gravity Device for Delivering Solutions at Constant Rate Intravenously, *J. LAB. & CLIN. MED.* 15: 556, 1930.
6. Jacobs, H. R.: An Apparatus for Constant Intravenous Injection Into Unrestrained Animals, *J. LAB. & CLIN. MED.* 16: 901, 1931.
7. Miller, B. F., and Van Slyke, D. D.: A Direct Microtitration Method for the Determination of Sugar in Blood, *J. Biol. Chem.* 114: 533, 1936.
8. Cole, W. H., and Womack, N. A.: A Simple and Inexpensive Apparatus for the Determination of the Metabolic Rate in Guinea Pigs, *J. LAB. & CLIN. MED.* 19: 540, 1934.
9. Jacobs, H. R.: A Practical Method for the Continuous Administration of Fluid Intravenously, *J. LAB. & CLIN. MED.* 19: 768, 1934.
10. Soskin, S.: A Simple Constant Injection Apparatus, *J. LAB. & CLIN. MED.* 18: 420, 1933.

## IMBEDDING OF THE SEMINAL FLUID\*

## A CONTRIBUTION TO THE STUDY OF THE MORPHOLOGY OF SEMEN

K. JOËL, M.D., JERUSALEM, PALESTINE

IT WAS possible to obtain a closer insight into the morphology of the semen only by the introduction of staining methods (Moench,<sup>1</sup> Cary and Hotchkiss,<sup>2</sup> Stiasny and Generales,<sup>3</sup> Michael and Joël<sup>4</sup>). Most of the investigators, however, studied the spermatozoa exclusively without considering the cellular factors of the semen. Only a few authors, as Fuerbringer and Waldeyer, referred to the cells which were found in the semen as "indifferent" testicular cells. Recently, however, Michael and Joël<sup>4</sup> proved from the study of a large number of seminal fluids that the cells found in these fluids were part of spermatogenesis. Moreover, they could observe the phenomenon of spermiophagia in the vital preparation as well as in the smear. In addition, the determination of the number of cells, their types, and their nature with simultaneous consideration of number, motility, and nature of the spermatozoa, enabled a differential diagnosis to be established from the ejaculate between degenerative-regenerative and atrophic changes of the testis on the one hand, and the degenerative changes of the epididymis on the other.

The smear method, however, was not sufficient for an exact study of the morphology of the semen, and this gave rise to the idea that we could obtain clearer pictures of the structure of the cells by imbedding the seminal fluid in paraffin.

The following is a description of the method I have worked out: The semen, used at the most a half to one hour after ejaculation, is centrifuged for twenty minutes (at 3000 rotations per minute). The supernatant fluid is poured away.

\*From the Laboratory of the Gynecological-Obstetrical Department of the Rothschild Hadassah Hospital, Jerusalem.

Received for publication, August 11, 1938.

and the centrifugate is kept for forty eight hours in a test tube with 4 per cent formalin, which should be changed after twelve and twenty four hours. The ejaculate is now somewhat firmer in shape. The formalin is then decanted, 50 per cent alcohol is added for twenty four hours, for the next twenty-four hours, 60 per cent alcohol, and so on with increasing concentration, each time 10 per cent more concentrated for 24 hours, respectively. The absolute alcohol, however, is allowed to stand for forty-eight hours, during which period it should be changed twice. Now the ejaculate, which formerly seemed to consist of crumbs, has been transformed into a uniform mass.

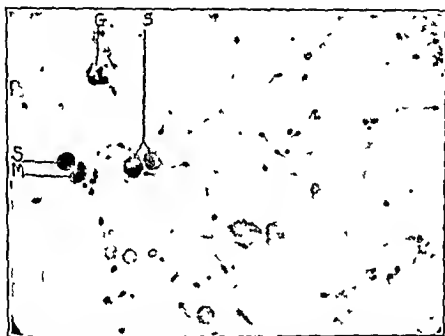


Fig 1—Smear showing spermatozoa in which head, neck and tail are distinctly recognizable. In addition there are 5 cells 3 of which are spermocytes (S) in different states of division, a macrophage (M) and a giant cell (G) are also present. The smear most distinctly shows the contours of the spermatozoa. Oil immersion magnified 500X.

The preparation should now be put into xylol alcohol in decreasing concentration for one-half to one hour:

9 parts alcohol + 1 part xylol
8 parts alcohol + 2 parts xylol
7 parts alcohol + 3 parts xylol
6 parts alcohol + 4 parts xylol
5 parts alcohol + 5 parts xylol
4 parts alcohol + 6 parts xylol
3 parts alcohol + 7 parts xylol
2 parts alcohol + 8 parts xylol
1 part alcohol + 9 parts xylol

The material is then put into xylol paraffin for half an hour and then placed in the incubator at 58° C for three hours in soft paraffin with a melting point of 54° C., and for three hours longer in hard paraffin with a melting point of 60° C. Finally, the semen is imbedded into the same paraffin.

After this is completed, the block is cut into sections 2 to 3 microns thick, if possible as serial sections. The sections are kept in the incubator for twenty-four hours at 38° C. The preparations are stained with haemalum eosin, triacid, and iron hematoxylin.

The advantages of this method are of scientific as well as of practical nature. On the one hand, this method enables us to gain insight into the finest

structure of the spermatozoa as well as the other cellular factors of the ejaculate and it gives a concentrated visual picture of the nuclear matter. On the other hand, from a practical aspect, the abundance of cells found in such preparations enables therapeutic effects to be studied, since, according to our experience, therapeutic effects in the beginning cannot be judged by the spermatozoa themselves but by the regenerative processes of the preliminary cellular stages.

Moreover, the imbedding method enables the differential diagnosis between aspermia and azoospermia to be established.

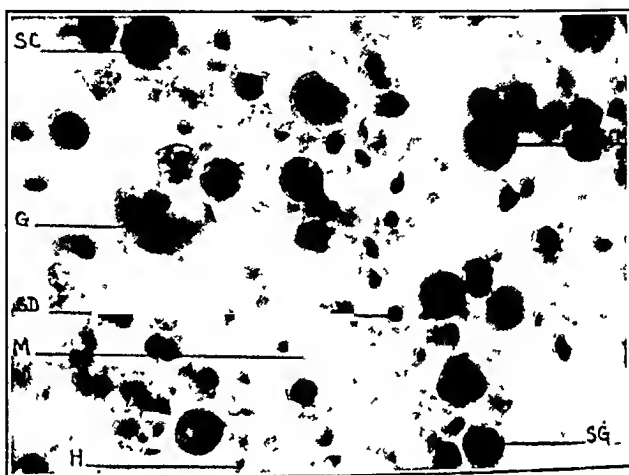


Fig. 2—Imbedded preparation. The tails have been lost owing to centrifugation and only the heads are seen, which are small, round, sometimes comma-shaped (*H*). The photomicrograph demonstrates the presence of abundant cellular substances, particularly cells of spermiogenesis. The large cells are macrophages (*M*). There are, in addition, spermatogonia (*SG*), spermatocytes in the state of quiescence and division (*SC* and *SCM*), spermides (*SD*) and giant cells (*G*). The cellular character of the ejaculate is particularly well demonstrated. Oil immersion, magnified 900X.

**Aspermia:** Absence of spermatozoa and their cellular precursors is produced by a high degree of testicular atrophy or by obstruction of the seminal canals. The smear, in this case, does not contain anything but some mucus and a few prostate crystals. The paraffin preparation shows the same picture.

**Azoospermia:** Absence of spermatozoa, but presence of their early cellular forms. This condition is brought about by partial atrophy of the testis. In the smear we find some scattered cells of spermiogenesis, but nowhere spermatozoa. In the imbedded preparation there are many more cells owing to centrifugation and the morphology of the cells becomes much more obvious.

These differences in cellular findings, which are very evident in our method help us greatly in differentiating between aspermia and azoospermia.

The difference between the diagnostic value of the smear, and that of the imbedded preparation is made obvious by the enclosed photomicrographs.

#### REFERENCES

1. Moench, G. L.: Studien zur Fertilität, Stuttgart, 1931, F. Enke.
2. Cary, W. H., and Hotchkiss, R. S.: J. A. M. A. 102: 587, 1934.
3. Stiasny, A. H., and Genzales, K. D. J., Jr.: Erbkrankheit und Fertilität, Stuttgart, 1937, F. Enke.
4. Michael, M., and Joël, K.: Zellformen im normalen und pathologischen Ejakulaten und ihre klinische Bedeutung, Schweiz. med. Wochenschr. 67: 757, 1937.

# LAUGHLEN TEST FOR SYPHILIS COMPARED WITH THE WASSERMANN AND KAHN\*

MAURICE R. MOOR, B A, M D C M, NORWICH CONN

IN AUGUST, 1935,<sup>1</sup> a new rapid agglutination method was described as a test for syphilis. This new procedure was considered to possess numerous outstanding advantages.<sup>1</sup> It is more rapid and easier to perform. The readings are more distinct because they depend upon an agglutination of colored particles in an unstained medium. Since it resembles the methods employed in typing blood, hospital interns and others are familiar with the technique. As the reagent is stable for at least several weeks it is ready for use in emergencies. It affords a convenient means of grading the degrees of positivity. Accuracy has not been sacrificed to secure speed or simplicity. The test requires only small amounts of material.

These advantages would attract anyone who assumes the responsibilities of a clinical laboratory, this is particularly true when one recalls that this method was first described on unactivated sera, a time saving feature later discarded.

It was decided to compare this test with the Kahn and Kolmer Wassermann tests.

Two technicians did all the tests in order to keep the personal element of difference at a minimum. The reagent was purchased from the Lederle Co.,

TABLE I  
UNACTIVATED SERUM

KAHN LAUGHLEN			KAHN LAUGHLEN			KAHN LAUGHLEN			KAHN LAUGHLEN		
Pos 4+	s p *	64	Pos 4+	w p †	25	Pos 4+	Quest	6	Pos 4+	Neg	16
Pos 3+	s p	2	Pos 3	w p	0	Pos 3+	Quest	1	Pos 3+	Neg	5
Quest	s p	9	Quest	w p	12	Quest	Quest	3	Quest	Neg	14
Neg	s p	12	Neg	w p	12	Neg	Quest	31	Neg	Neg	202
Totals		87			49			41			237

\*s p = strongly positive

†w p = weakly positive

TABLE II  
INACTIVATED SERUM

KAHN LAUGHLEN			KAHN LAUGHLEN			KAHN LAUGHLEN			KAHN LAUGHLEN		
Pos 4+	s p	1	Pos 4+	w p	5	Pos 4+	Quest	0	Pos 4+	Neg	2
Pos 3+	s p	0	Pos 3+	w p	1	Pos 3+	Quest	0	Pos 3+	Neg	2
Quest	s p	0	Quest	w p	1	Quest	Quest	0	Quest	Neg	1
Neg	s p	4	Neg	w p	4	Neg	Quest	2	Neg	Neg	107
Totals		5			11			2			112

\*From the Laboratory of the William W. Backus Hospital, Norwich.  
Received for publication September 2, 1938.

New York, and the instructions submitted with the antigen were regarded carefully. At first we were advised to use unactivated serum; later inactivation was recommended. This is indicated in each table.

TABLE III  
UNACTIVATED SERUM

WASS. LAUGHLIN			WASS. LAUGHLIN			WASS. LAUGHLIN			WASS. LAUGHLIN		
Pos. 4+	s.p.	50	Pos. 4+	w.p.	22	Pos. 4+	Quest.	6	Pos. 4+	Neg.	13
Pos. 3+	s.p.	2	Pos. 3+	w.p.	3	Pos. 3+	Quest.	0	Pos. 3+	Neg.	2
Pos. 2+	s.p.	2	Pos. 2+	w.p.	0	Pos. 2+	Quest.	1	Pos. 2+	Neg.	3
Quest.	s.p.	0	Quest.	w.p.	3	Quest.	Quest.	1	Quest.	Neg.	0
Neg.	s.p.	2	Neg.	w.p.	8	Neg.	Quest.	2	Neg.	Neg.	64
Totals		56			36			10			82

TABLE IV  
UNACTIVATED SERUM

WASS. KAHN LAUGHLIN				WASS. KAHN LAUGHLIN				WASS. KAHN LAUGHLIN			
Pos. 4+	Pos. 4+	s.p.	49	Pos. 4+	Pos. 4+	w.p.	19	Pos. 4+	Pos. 4+	Quest.	3
Pos. 3+	Pos. 4+	s.p.	1	Pos. 3+	Pos. 4+	w.p.	2	Pos. 2+	Pos. 4+	Quest.	1
Pos. 2+	Pos. 4+	s.p.	1								
Neg.	Pos. 4+	s.p.	1	Quest.	Pos. 4+	w.p.	1	Quest.	Pos. 4+	Quest.	1
				Neg.	Pos. 4+	w.p.	2	Quest.	Pos. 4+	Quest.	1
				Neg.	Pos. 3+	w.p.	2	Neg.	Pos. 4+	Quest.	0
Pos. 4+	Neg.	s.p.	1	Pos. 4+	Quest.	w.p.	2	Pos. 3+	Pos. 3+	Quest.	0
Pos. 3+	Quest.	s.p.	1	Pos. 3+	Quest.	w.p.	1	Pos. 2+	Pos. 3+	Quest.	0
Pos. 2+	Quest.	s.p.	1	Pos. 2+	Quest.	w.p.	1				
				Quest.	Quest.	w.p.	2	Neg.	Pos. 3+	Quest.	0
				Neg.	Quest.	w.p.	2	Pos. 4+	Quest.	Quest.	2
				Quest.	Neg.	w.p.	1	Neg.	Quest.	Quest.	0
				Pos. 4+	Neg.	w.p.	1	Pos. 4+	Neg.	Quest.	0
				Quest.	Neg.	w.p.	1	Pos. 3+	Neg.	Quest.	0
				Neg.	Neg.	w.p.	5	Neg.	Neg.	Quest.	2
Totals		55				42				10	82

#### DISCUSSION

Tables I-IV are divided into columns based upon the practical values of this work.

Four hundred and fourteen unactivated sera (Table I) were tested by the Laughlen and the Kahn methods. The bloods were from new admission cases for diagnosis and treatment control patients.

21.98 per cent (91) gave a definitely positive reaction with the Kahn and Laughlen tests.

60.3 per cent (250) gave a definitely negative or questionable reaction with the Kahn and Laughlen tests.

10.86 per cent (45) gave a definitely negative or questionable reaction with the Kahn; and a definitely positive reaction with the Laughlen.

7.2 per cent (28) gave the Kahn definitely positive and the Laughlen definitely negative or questionable.

These figures show that the two tests agree in 82 per cent of the series, and an 18 per cent error by the Laughlin if we are to accept the time honored Kahn tests as a substandard

It was while our work was being done that we were advised to inactivate the serum The introduction of this step automatically canceled the one great advantage of the Laughlin test However it was decided to run a smaller series on inactivated serum, 130 sera (Table II) were tested, and these were obtained from new admission patients

- 53.8 per cent (7) shows the Kahn and Laughlin definitely positive
- 84.6 per cent (110) shows the Kahn and Laughlin questionable or negative
- 6.9 per cent (9) shows the Kahn is negative and the Laughlin definitely positive
- 3.07 per cent (4) shows the Kahn definitely positive and the Laughlin questionable or negative

The accuracy of the Laughlin test as here judged by the Kahn, was increased by inactivating the sera The two tests gave the same reactions in 90 per cent and definitely disagreed in 10 per cent of the tests This is an improvement of 8 per cent over the unactivated series

- 184 sera (Table III) were submitted to the Wassermann and Laughlin tests, using unactivated serum for the Laughlin test
- 42.9 per cent (79) showed the Wassermann and Laughlin definitely positive
- 7.06 per cent (13) showed the Wassermann questionable or negative and the Laughlin definitely positive
- 13.5 per cent (25) showed the Wassermann definitely positive and the Laughlin questionable or negative
- 36.4 per cent (67) showed the Wassermann and Laughlin questionable or negative

In comparison with the Wassermann reaction, the Laughlin test was erroneous in 20.56 per cent of 184 tests

A series of 189 sera (Table IV) were tested by the Wassermann, Kahn, and Laughlin tests, using unactivated serum for the Laughlin

- 7.8 per cent (72) gave the Wassermann Kahn and Laughlin definitely positive
- 10.05 per cent (19) gave the Wassermann and Kahn definitely positive and the Laughlin is questionable or negative
- 7.17 per cent (6) gave the Wassermann questionable, the Kahn definitely positive, and the Laughlin definitely positive
- 2.1 per cent (4) gave the Wassermann questionable, the Kahn definitely positive, and the Laughlin questionable or negative
- 4.2 per cent (8) gave the Wassermann positive, the Kahn negative or questionable, and the Laughlin definitely positive
- 2.6 per cent (5) gave the Wassermann positive, the Kahn negative or questionable, and the Laughlin questionable or negative
- 5.8 per cent (11) gave the Wassermann and the Kahn negative or questionable and the Laughlin definitely positive
- 3.8 per cent (64) gave the Wassermann negative or questionable, the Kahn and Laughlin negative or questionable

Table IV shows the Wassermann, Kahn, and Laughlen tests agreeing in 71 per cent (136 sera); in 15.87 per cent (30 sera) the Wassermann and Kahn have the same reaction, and the Laughlen test is definitely disagreeing with both. The other 12.33 per cent (23 sera) gives varied findings as tabulated, and their numbers are too few to be considered valuable.

#### SUMMARY

The Laughlen test, carried out in uninactivated sera, shows an error of 18.66 per cent compared with the Kahn; of 20.56 per cent compared with the Kolmer-Wassermann, and of 15.87 per cent compared with the Kolmer-Wassermann and Kahn. On inactivated sera compared with the Kahn there was an error of 10 per cent.

#### CONCLUSION

The Laughlen agglutination test for syphilis compared with the Kolmer-Wassermann and Kahn shows results too erroneous to justify the recommendations of its use in its present form.

#### REFERENCE

1. Laughlen, G. F.: A Rapid Test for Syphilis, *Canad. M. A. J.* 33: 179, 1935.

## MICROMETHODS FOR THE QUANTITATIVE ANALYSIS OF URINARY CALCULI\*

HERMAN BROWN, B.S., PHILADELPHIA, PA.

IN THE course of an investigation into the composition of the various strata of urinary calculi, the need was felt for a scheme of analyses which would yield quantitative data when working with very small samples. Not only are some urethral and ureteral stones quite small, but when attempts were made to secure quantitative data on the nucleus and shell separately, the usual macromethods were found to be inapplicable to the amounts of material available.

In the methods detailed in this paper recourse was had to the principles involved in blood chemistry. These adaptations of existing blood micro-methods were checked against the usual macromethods, using mostly vesical calculi which are usually large enough to provide sufficient material. In general, the agreement was quite good; differences greater than 2 per cent were the exception rather than the rule. The scheme outlined below has the following advantages over the usual macromethods: (1) Speed; in a laboratory in which an eight cup centrifuge is available, eight samples may be quantitatively analyzed for calcium, magnesium, total nitrogen, ammonium, phosphorus, uric acid, oxalic acid, creatinine, and carbonic acid in less than three days. (2) Convenience; all the reagents, standards, and apparatus in common use in the average biochemical laboratory may be used without modification.

\*From the Research Institute of Cutaneous Medicine, Philadelphia.  
Received for publication, September 8, 1938.

Only a recalculation of the standard equivalents from element to anhydride is necessary (3) The above nine determinations may be made on a 50 mg sample if an ordinary analytical balance is used. If a microbalance is available, much smaller samples can be used.

*Preparation of Sample*—The various layers of many urinary calculi may readily be separated from each other and from the nucleus by manipulation with forceps and scissors. To cut the larger stones, a small bone cutting saw is of advantage. In those calculi in which the various layers are poorly defined a good sample of the outer layer may be obtained by simply scraping the outer surface, and the sample corresponding to the nucleus obtained by digging out a small portion from the approximate center of the end surfaces. Loss of sample will be avoided if the stone be tightly wrapped in white paper before sawing. Each sample is ground to a fine powder in a mortar.

*Moisture*—The powdered sample is weighed in a small tared test tube and dried in an oven overnight at  $104^{\circ}$  to  $105^{\circ}$  C. The loss in weight represents moisture.

*Calcium Oxide*—Twelve and a half milligrams of dried powder are placed in a pyrex test tube (100 by 25 mm), and 0.8 cc concentrated sulfuric acid is added. The test tube is then heated over a microburner until maximum charring is attained, it is then allowed to cool somewhat and 3 to 5 drops of superoxol are cautiously added. Heating is then continued until a water-clear solution is obtained. The entire digestion requires about three minutes. The contents of the tube is then transferred quantitatively to a 25 cc volumetric flask, made up to the mark with distilled water and mixed.

To 5 cc of the solution in a 50 cc conical centrifuge tube are added, in order, 1 cc of 4 per cent ammonium oxalate, 3 drops of methyl red, strong ammonia water to faint alkalinity, and 5 per cent acetic acid to faint acidity. After standing two hours the precipitate is centrifuged down and the supernatant solution is carefully poured into another 50 cc conical centrifuge tube to be used in the magnesium determination. In transferring this solution, the tube is allowed to drain a moment and the tip rinsed into the other centrifuge tube by means of a fine stream of water from the wash bottle. The precipitate of calcium oxalate is washed and titrated after the manner of Clark and Collip<sup>1</sup>. Titration may be carried out directly in the centrifuge tube after solution in normal sulfuric acid (1 cc 0.01 N  $\text{KMnO}_4 = 0.28$  mg  $\text{CaO}$ ). (The percentage calcium oxide in dried powder equals number cubic centimeters of 0.01 normal potassium permanganate times 11.2.)

*Magnesium Oxide*—To the supernatant solution from the calcium determination is added 1 cc of 5 per cent ammonium phosphate, strong ammonia water dropwise to alkalinity, and then 0.5 cc in excess. The centrifuge tube is allowed to remain overnight and then treated according to the Denis method for blood magnesium. The magnesium standard for blood containing 0.02 mg of magnesium per 10 cc is equivalent to 0.0331 mg magnesium oxide per 10 cc. Colorimeter settings may have to be varied somewhat to accommodate the varying amounts of magnesium in the calculi, but we have found that the magnesium content of the majority of calculi comes within the color range of this standard.



*Phosphorous Pentoxide.*—The method of Fiske and Subbarow<sup>3</sup> is applied to 1 c.c. of the above acid digest. This is placed in a test tube graduated at 10 c.c., diluted with about 4 c.c. of water, and treated exactly as a blood filtrate. The blood phosphate standard commonly used (5 c.c. = 0.4 mg. phosphorus) is equivalent to 0.914 mg. phosphorous pentoxide per 5 c.c. The color range with this method is closely proportional to the phosphorous content, and the 1 c.c. sample used permits the phosphorous determination of widely varying samples without repeating on more or less sample.

*Total Nitrogen.*—Other than ammonium salts the determination of total nitrogen is roughly a measure of the amount of uric acid to be expected and is helpful in determining the amount of uric acid standard to use in the latter determination. The total nitrogen is determined on a 5 c.c. sample of the above digest which is carefully neutralized to remove interfering phosphates (centrifuge) and treated exactly as in the ammonium nitrogen determination described below. It is of advantage to run both determinations simultaneously, since the same standard ammonium sulfate solution suffices for both tests.

*Uric Acid.*—To 12.5 mg. of dried stone powder in a 50 c.c. test tube, are added about 40 c.c. of 0.2 per cent lithium carbonate and the contents heated on a steam bath for one hour, with occasional shaking. The solution is filtered hot into a 50 c.c. volumetric flask, the test tube being rinsed with a little hot water. The filtrate is allowed to cool and made up to the mark with water. One cubic centimeter is used for the determination, being diluted to 15 c.c. with water, and followed by Brown's<sup>4</sup> procedure. The usual uric acid blood standard is used from which three comparison standards are prepared using 5, 10, and 15 c.c. of standard solution. These standards cover a uric acid content equivalent to 5-60 per cent of the calculi, a range which will cover nearly all stones. If stronger colors are encountered, the test may be repeated using 0.5 c.c. of the above filtrate.

*Oxalate.*—Twelve and a half milligrams of dried stone powder are heated on the steam bath with about 20 c.c. of approximately normal sulfuric acid. After one to two hours' digestion, the solution is filtered hot into a 25 c.c. volumetric flask, and the undissolved portion washed with a little hot water. The filtrate is cooled to room temperature and diluted to the mark. In the absence of uric acid the oxalate in the filtrate is determined directly. Five cubic centimeters of filtrate are warmed to 60°-80° C. and titrated with 0.01 N potassium permanganate (1 c.c. of 0.01 N  $\text{KMnO}_4$  = 0.36 mg.  $\text{C}_2\text{O}_3$ ). Because of the reducing action of uric acid on potassium permanganate, it is better to remove the oxalate as calcium oxalate if much uric acid is present. Five cubic centimeters of filtrate in a 50 c.c. centrifuge tube are neutralized and made acid with acetic acid as in the calcium determination previously outlined, and the oxalate precipitated by the addition of 0.5 c.c. of 10 per cent calcium chloride. After standing two hours the precipitate is treated exactly as in the calcium determination, except that the factor for oxalate is used in the calculation.

*Ammonium.*—Fifteen cubic centimeters of the above normal acid extract are placed in a 25 c.c. graduated cylinder and carefully neutralized with 10 per

cent sodium hydroxide. Water is added to the 20 cc mark and the precipitate (phosphate) removed by centrifuging. Ten cubic centimeters of the clear supernatant fluid (equivalent to 7.5 cc of original filtrate) are placed in a 50 cc volumetric flask, about 20 cc of water and 2 drops of 1 per cent gum ghatti are added, and then 5 cc of Nessler's solution\*. Dilute to the mark and compare the resulting color with that produced by 3 cc of the usual ammonium sulfate standard in a 100 cc volumetric flask which has been simultaneously treated with 10 cc of Nessler's solution (3 cc ammonium sulfate standard = 0.3 mg of N or 0.386  $\text{NH}_4$ ).

*Creatinine*—Pour the remainder of the above supernatant fluid into a 25 cc graduated mixing cylinder and dilute to 10 cc. Add 5 cc of Jaffe's reagent,<sup>6</sup> mix, and compare after fifteen minutes with 10 cc of water to which has been added 5 cc of alkaline picrat. If the unknown exhibits more color than that produced by the alkaline picrat in water then the creatinine present is determined by colorimetric comparison with a standard creatinine solution simultaneously treated with alkaline picrat exactly as in the blood creatinine determination.<sup>6</sup>

*Carbonate*—Since many calculi have been found free of carbonate a qualitative test is first made. A few grains of powder stone are suspended in a drop of water on a microscope slide covered with a cover glass and, while being observed through the low power objective, a drop of hydrochloric acid is placed at one edge of the cover glass. As the acid seeps under the cover glass, bubbles of gas will be seen to form if carbonate is present. To determine the amount of carbonate present the following method, a modification of Newcomb's,<sup>7</sup> is utilized.

A long stemmed open end bell shaped separatory funnel having a well fitting tap (Fig 1), is used. The 50 cc size has a stem of sufficient size and bore (about 300 by 5 mm). Beginning with the stopcock end the stem is graduated into cubic centimeters which are then subdivided into tenths. A stem of the above size can be graduated into 5 cc with the subdivisions sufficiently spaced, readily to permit of estimations to 0.05 cc. The stem is connected by means of a length of nitrometer tubing to a straight adapter which is used in place of the conventional leveling bulb. The use of an adapter makes for more accurate adjustment of the mercury level. By means of a copper wire fixed to the adapter its height may be easily and accurately adjusted and fixed by simply wrapping the loose end of the copper wire around a ring stand clamp.

The only other piece of equipment needed is a small capsule used for introducing the sample. This is readily made from a piece of 10 mm thin walled glass tubing. The tubing is collapsed in the flame about 2 cm from one end, and the glass then drawn down sufficiently small to fit into the bore of the stopcock (Fig 1A).

The leveling bulb is filled with enough mercury to allow some overflow above the stopcock of the separatory funnel. About 10 mg of stone powder

are accurately weighed into the tared capsule, the powder being placed on the side forming the shoulder. The tap of the separatory funnel is opened, and the mercury level is adjusted so that the mercury is about 0.2 c.c. below the level of the tap. The separatory funnel is clamped at an angle of about  $15^\circ$  to the vertical. The drawn out end of the capsule is carefully inserted into the bore of the stopcock, the separatory funnel is reset to a vertical position, and the sample of powdered stone introduced into the stem by gentle tapping and prodding with a fine platinum wire. After most of the powder

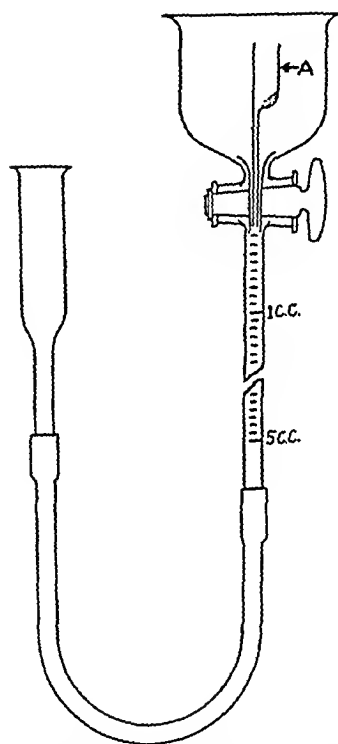


Fig. 1.—Separatory funnel with graduated stem used in determination of carbon dioxide in small amounts of powdered sample. The capsule for introducing the sample is shown at A.

has been introduced, the capsule with the adherent powder is withdrawn and reweighed. Sufficient distilled water is now added to fill the bore of the stopcock, the latter closed, and the excess water drawn off. About 0.3 c.c. of 1:1 hydrochloric acid is now placed in the funnel above the stopcock and the mercury level lowered about 0.5 c.c. The stopcock is cautiously opened to allow about 0.2 c.c. of acid to run into the stem, after which it is quickly closed. The gas as it is liberated is maintained under approximately atmospheric pressure by lowering the leveling bulb so that the mercury levels are always nearly the same. After completion of the reaction, the acid water in the stem is saturated with carbon dioxide by manipulating the leveling bulb to produce an alternate rise and fall of the mercury level in the stem of 0.5 to 1.0 c.c. The leveling bulb is then adjusted so that the gas in the stem is brought to atmospheric pressure and the volumes of both gas and liquid are

read off from the stem. The barometric pressure and temperature are noted, after which the weight of carbon dioxide may be calculated from the usual formula:

$$W = 0.706 \times \frac{(P - P')}{273 + t} \times (B + 0.85 \times A)$$

Where W = Weight in milligrams of carbon dioxide

P = Barometric pressure in millimeters

P' = Vapor tension of water at t° C

t = Room temperature in Centigrade

B = Volume of gas in c.c.

A = Volume of acid water in c.c.

The value 0.85 represents the average amount of gas dissolved in the acid water at usual room temperatures. If the total volume of acid water is kept below 0.4 c.c., this value may be used over a fairly wide room temperature range without introducing any serious error. The weight of carbon dioxide may, of course, also be taken directly from standard tables without any calculation other than that required to determine the amount of carbon dioxide dissolved in the acid water, i.e.,  $0.85 \times A$ .

#### CONCLUSIONS

A systematic scheme of quantitative analysis of urinary calculi is presented.

Methods for the determination of calcium, magnesium, total nitrogen, ammonium, phosphorus, uric acid, oxalic acid, creatinine, and carbonic acid are described.

These nine determinations may be made on less than 50 mg. of sample with materials and reagents ordinarily used in a biochemical laboratory equipped for blood chemistry.

#### REFERENCES

1. Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium With a Suggested Modification, *J. Biol. Chem.* 63: 461, 1925.
2. Denis, W.: The Determination of Magnesium in Blood, Plasma and Serum, *J. Biol. Chem.* 52: 411, 1922.
3. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorous, *J. Biol. Chem.* 66: 375, 1925.
4. Brown, H.: The Determination of Uric Acid in Blood, *J. Biol. Chem.* 68: 123, 1926.
5. Kolmer, J. A., and Boeiner, F.: *Approved Laboratory Technique*, ed. 2, New York, 1928, Appleton Century Co., p. 720.
6. Folin, O., and Wu, H.: A System of Blood Analyses, *J. Biol. Chem.* 38: 81, 1919.
7. Newcomb, C.: A Scheme for Analysis of Small Urinary Calculi, *Indian J. M. Research* 17: 735, 1930.

## FURTHER OBSERVATIONS ON THE DETERMINATION OF SULFANILAMIDE IN BLOOD\*

E. G. SCHMIDT, PH.D., BALTIMORE, MD.

IN A previous paper<sup>1</sup> from this laboratory it was shown that the sulfanilamide content of tungstic acid blood filtrates can be readily determined by means of sodium-beta-naphthoquinone-4-sulfonate. Further experience with this method has revealed the fact that it does not give quantitative recovery in that 10 to 15 per cent of the sulfanilamide is lost during deproteinization. This loss seems to vary somewhat with the temperature, and ranges from 10 per cent during warm summer months to about 15 per cent during the winter when the room temperature averages about 20° F. lower. We are unable to explain why this discrepancy was not observed during the preliminary experimentation. Seudi<sup>2</sup> has likewise observed a loss of sulfanilamide during the removal of blood proteins.

### METHODS

The data on the new recovery experiments performed during the last summer and reported in Table I were secured by adding to 18 c.c. portions of pooled, oxalated, human blood, 2 c.c. quantities of stock sulfanilamide (Winthrop Chemical Co., Inc.) solutions of such strengths as to yield a series of bloods containing 2.5 to 20 mg. sulfanilamide per 100 c.c. The sulfanilamide-containing bloods were mixed, and four different filtrates, namely, tungstic acid filtrate,<sup>3</sup> p-toluenesulfonic acid, trichloroacetic acid, and alcoholic filtrates of Marshall,<sup>4-7</sup> were prepared by careful deproteinization of exactly 3 c.c. portions of blood.

The tungstic acid filtrates were analyzed by the naphthoquinonesulfonic acid method, as previously described by the author.<sup>1</sup> Since increased acidity depresses color production, both standards and filtrate must be at the same pH. The addition of one drop of 0.1 normal hydrochloric acid to each brings them to a pH of about 3.2, which seems to constitute a satisfactory reaction medium. More than one drop of acid is undesirable. Light destroys the quinone reagent, especially in the standards. Hence the colorimetric readings should be made within ten minutes after removal from the dark, particularly when specimens containing but small quantities of drug are being analyzed. Ordinary tungstic acid filtrates contain no factor, other than their acidity, which might influence color production. Dilution of stock sulfanilamide solutions with equal quantities of either water or control tungstic acid blood filtrate, after addition of the acid, always resulted in equal color production with the naphthoquinonesulfonic acid reagent. In each case a sample of the

\*From the Department of Biological Chemistry, University of Maryland, School of Medicine, Baltimore.

Received for publication, September 15, 1938.



TABLE II

COMPARATIVE ANALYSIS OF BLOOD OF PATIENTS UNDERGOING SULFANILAMIDE THERAPY

CASE NO.	SULFANILAMIDE FOUND BY MARSHALL'S METHOD			SULFANILAMIDE FOUND BY NAPHTHOQUINONE-SULFONIC ACID METHOD ON TUNGSTIC ACID FILTRATES		DIAGNOSIS
	ON P-TOLU-ENESULFONIC ACID FILTRATES	ON TRICHLOR-ACETIC ACID FILTRATES	ON ALCOHOLIC FILTRATES	mg./100 c.c.	mg./100 c.c.*	
1	17.00	16.7	17.0	15.0	16.67	Meningitis, <i>Streptococcus viridans</i>
2	15.7	14.8	16.7	13.64	15.16	Meningitis, <i>Streptococcus viridans</i>
3	10.26	10.0	10.36	9.18	10.2	Undulant fever
4	10.10	9.6	10.70	8.91	9.9	Undulant fever
5	8.17	8.23	8.70	7.19	7.99	Bronchopneumonia
6	6.31	6.25		5.81	6.45	Salpingitis
7	6.97	6.70		6.15	6.83	Septicemia
8	6.97	6.60	6.90	6.15	6.83	Empyema
9	5.94		6.06	5.61	6.23	Pyelitis of pregnancy
10	5.62	5.13	5.65	4.95	5.5	Pyelitis of pregnancy
11	4.35	4.10	4.56	4.00	4.44	Pregnancy
12	3.95	3.86	4.20	3.66	4.07	Pyelitis

\*The obtained value divided by the correction factor 0.9.

control blood was tested in order to eliminate sulfanilamide contamination. It was particularly helpful to discover that a 0.5 per cent solution of sodium-beta-naphthoquinone-4-sulfonate (Eastman No. 1372) can be preserved unchanged in a stoppered flask for at least a month if kept cold and in the dark. A quantity of reagent sufficient for daily use can be prepared by simply diluting 1 c.c. to 10 c.c. with water, thus saving time and material. Obviously, since the reagent itself is yellow, the determination will be valueless unless both standard and unknown filtrate match each other within 2 mm. in the colorimeter. Therefore, unless the approximate value is known in advance, several standards must be set up for each determination.

The diazotization procedures were carried out as outlined by Marshall and his co-workers in their various papers.<sup>4-7</sup> The blood proteins precipitated by the alcohol were filtered off by being passed twice through double filter paper. The standard solutions were filtered in a similar manner in order to keep this factor constant. In addition, 5 c.c. of the alcoholic reagent (1 c.c. of activated dimethyl-alpha-naphthylamine in 249 c.c. alcohol) were used in each procedure. In our hands unheated specimens of dimethyl-alpha-naphthylamine (Eastman No. 1060) gave low sulfanilamide values for the p-toluenesulfonic acid (Eastman No. 984) and trichloroacetic acid blood filtrates, because these filtrates tend to retard or inhibit color development. Consequently, color development in the standards was more rapid—hence low values were usually obtained. Activation of this reagent by heating, as Marshall<sup>6</sup> has shown, and the addition of the phosphate buffer and the ammonium sulfanate (La Motte) improved markedly the sulfanilamide recovery.<sup>7</sup> All colorimetric readings for these procedures were made about ten minutes after the addition of the last reagent. Other workers have also used trichloroacetic acid filtrates for blood sulfanilamide analysis.<sup>8, 9</sup>

## CONCLUSION AND SUMMARY

The data in Table I show that good recovery of added sulfanilamide can be secured with two of the Marshall procedures, but that the alcoholic filtrate method, in our hands at least, frequently gives slightly higher values. The data also show that the naphthoquinonesulfonic acid method on tungstic acid filtrates yields values which average about 10 per cent too low. Since this loss tends to be somewhat higher in cold weather (about 15 per cent), each laboratory should probably determine its own correction factor. Our method for the determination of sulfanilamide in cerebrospinal fluid has eliminated filtrate preparation—hence no correction factor is involved in this procedure.<sup>10</sup>

In Table II are given data on the comparative analysis of a series of bloods from patients undergoing sulfanilamide therapy. It is evident that the values obtained by the naphthoquinonesulfonic acid method when divided by the correction factor 0.9, check well with the values obtained by the diazotization procedure on the p-toluenesulfonic acid and trichloroacetic acid filtrates. Again the alcoholic filtrate yields slightly higher values.

## REFERENCES

- 1 Schmidt, E. G. The Determination of Sulfanilamide in Tungstic Acid Blood Filtrates by Means of Sodium Beta Naphthoquinone 4 Sulfonate. *J Biol Chem* 122 757, 1938.
- 2 Scudl, J. V. The Determination of Sulfanilamide (p-Amino benzenesulfonamide) in Biological Media, *J Biol Chem* 122 539, 1938.
- 3 Haden, R. L. A Modification of the Folin Wu Method for Measuring Protein Free Blood Filtrates. *J Biol Chem* 56 469, 1923.
- 4 Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C. p-Amino benzenesulfonamide, *J A M A* 108 953, 1927.
- 5 Marshall, E. K., Jr. Determination of Sulfanilamide in Blood and Urine, *Proc Soc Exper Biol & Med* 36 422, 1937.
- 6 Marshall, E. K., Jr. Determination of Sulfanilamide in Blood and Urine. *J Biol Chem* 122 263, 1937.
- 7 Marshall, E. K., Jr., and Litchfield, J. T., Jr. The Determination of Sulfanilamide, *Science* 88 85, 1938.
- 8 Proom, H. Estimation of Sulfanilamide in Blood and Other Body Fluids, *Lancet* 1 260, 1938.
- 9 Kamlet, J. The Determination of Sulfanilamide in Blood. *J LAB & CLIN MED* 23 1101, 1938.
- 10 Schmidt, E. G. The Determination of Sulfanilamide in Cerebrospinal Fluid by Means of Sodium Beta Naphthoquinone 4 Sulfonate, *J LAB & CLIN MED* 23 648, 1938.



## NOTES ON THE MICROSCOPIC DIAGNOSIS AND CULTURAL DIFFERENTIATION OF THE GONOCOCCUS\*

ALFRED COHN, M.D., NEW YORK, N. Y.

THE following report on the microscopic and cultural diagnosis of the gonococcus does not claim to present any basically new methods. Its twofold aim is (1) To report a method which simplifies the microscopic examination of Gram stained smears containing only a few intracellular gram-negative diplococci. (2) To emphasize the use of a 5 per cent horse blood plate as a diagnostic aid in differentiating the gonococcus from the meningococcus culture.

The simple procedure of the microscopic diagnosis of acute untreated gonorrhea is well known. However, in cases of subacute and chronic infection, with very few intracellular organisms, the diagnosis becomes difficult, especially if the Gram method is used with carbolfuchsin as a counterstain. Since the color of the microscopic organisms differs only little from that of the cytoplasm and the nuclear substance of the leucocytes, single intracellular diplococci may be overlooked easily, and the microscopic report issued as negative. In order to overcome these uncertainties Jacobsthal<sup>1</sup> recommends the use of a second smear stained with methylene green pyronine (Unna Pappenheim). He states that the Gram method is superior for finding gram-positive cocci, but inferior in revealing intracellular gram-negative cocci. In the event the Unna Pappenheim stain reveals intracellular diplococci, the examiner has to check this finding with the Gram stained smear. In addition to the Unna Pappenheim a third smear stained with methylene blue 1:2,000 has also been used in our laboratory for the same purpose.

In an attempt to simplify the procedure, to save time and material, another method has been tried. A 20 per cent solution of trichloroacetic acid, the use of which was previously recommended by Lanz<sup>2</sup> in smears stained with methylene blue and eosin, evidently replaces the second and third smear in subacute and chronic cases. The technical procedure is as follows: The material to be examined is taken with a loop from the genital organ and spread in a thin and even film on the slide. The smear is allowed to dry in the air. In lieu of fixing the smear by heat, it is then treated with a 20 per cent solution of trichloroacetic acid for one-half minute, at which time it assumes a whitish appearance. The acid is washed off with tap water, and the slide is dried between layers of absorbent paper. In contrast to the method for Gram stain previously described,<sup>3</sup> 1 per cent aqueous crystal violet, made up from a saturated alcoholic solution, is used. It was found that if the diluted dye is filtered when prepared, it will remain stable for at least two weeks. After one minute, the crystal violet is washed with Lugol's solution (iodine 1, potas-

\*From the Bureau of Laboratories, Department of Health, New York.  
Received for publication, September 17, 1933.

sium iodine 2, distilled water to make 100) until all precipitates have been removed. Fresh Lingol's solution is applied for one minute and the smear is decolorized with 95 per cent ethyl alcohol until the color ceases to run. The slide is now rinsed in tap water and blotted. As a counterstain the Ziehl-Neelsen carbolfuchsin, diluted 1:10 is used for thirty seconds. After rinsing in water and drying, the smear is ready for microscopic examination.

Comparison of Gram stained smears with and without previous treatment of trichloroacetic acid shows that the use of the acid makes the murens and the cells transparent. It is, therefore possible to find single pairs of gram negative cocci more easily within the cellular or nuclear substance. The use of the acid in clinical smears does not seem to cause gram positive microorganisms to become gram negative. It has occasionally been observed, however, that smears taken from cultures of streptococci and staphylococci and subjected to the treatment of trichloroacetic acid have the tendency to lose their gram positivity. Since it is unnecessary to use the acid in smears from cultures, this source of confusion may be disregarded.

The second object of this study is the differentiation of the gonococcus culture from that of the meningococcus. The growth and appearance of the colonies, the sugar fermentation, the agglutination and the alkali solubility tests are used for this purpose, but unfortunately these criteria do not always work satisfactorily. While the gonococcus regularly ferments dextrose according to the method previously described<sup>1</sup> there are a few strains of meningococci which do give a dextrose fermentation but do not affect the maltose. The agglutination and the alkali solubility tests do not always show clear cut results. Therefore, a need for an additional differential aid is apparent. For this purpose a 5 per cent horse blood plate may be employed, which the gonococcus, as a rule, does not hemolyze, while the meningococcus does after forty-eight hours of incubation at 37° C. In a series of many hundreds of examinations in which gonococci were identified no hemolysis has been found. However, testing 12 meningococcus strains of different ages (obtained through the courtesy of Miss L. Mishulow of the Health Department) showed hemolysis after forty-eight hours' incubation at 37° C. When washed cells are used in preparing the 5 per cent horse blood agar, hemolysis occurs after twenty-four hours' incubation. Rabbit blood, in our limited experiences is not hemolyzed by the meningococcus.

This hemolyzing quality of the meningococcus was first described by Gordon<sup>4</sup> in 1920, and years later by Levinthal<sup>5</sup> and Jorchimovits.<sup>6</sup> This observation has not been made use of generally. Our laboratory makes it a rule to identify a strain as a gonococcus by including culture on the 5 per cent horse blood agar plate. A gram negative diplococcus is identified as a gonococcus when it gives no growth, or only very slight growth, on the first transfer on plain agar, gives no hemolysis on blood agar, and ferments only dextrose.

#### SUMMARY

1. Preliminary treatment of the Gram stain with 20 per cent trichloroacetic acid is recommended in order to detect single intracellular gram negative diplococci.

2. Five per cent horse blood agar is one of the media used to differentiate the gonococcus and the meningococcus culture.

## REFERENCES

1. Jacobsthal, E.: Bemerkungen zur mikroskopischen Diagnose der Gonokokken und Meningokokken, *Dermat. Wehnschr.* 88: 101, 1929.
2. Lanz, A.: Ein neues Verfahren der Gonokokkenfärbung, *Deutsche med. Wehnschr.* 1934 S. 200.
3. Cohn, A.: The Importance of Bacteriological Culture for the Diagnosis of Gonorrheal Vulvovaginitis and Proctitis in Children, *Am. J. Syph., Gonorr. & Ven. Dis.* 20: 173, 1936.
4. Gordon, M. H.: Bacteriological Studies of Cerebrospinal Fever Among Troops (1914-1918). *Brit. M. J.* 2: 423, 1920.
5. Levinthal, W.: *Lehrbuch der Gonorrhoe* v. A. Buschke und E. Langer, 1926, page 105.
6. Joachimovits, R.: Die Hämolyse und Oxydasereaktion als Mittel zur Differentialdiagnose von Gonokokkus, Mikrokokus katarrhalis und Meningokokkus, *Zentralbl. f. Bakt.* (Abt. 1) 125: 240, 1932.

This work was made possible through the aid of the Milbank Memorial and the New York Foundations.

## A RAPID METHOD FOR DETERMINING HYPOPROTEINEMIA WITH ONE DROP OF SERUM\*

JONAS KAMLET, BROOKLYN, N. Y.

**B**ING has described a rapid method for determining hypoproteinemia by floating a glass bead of specific gravity 1.0245 in serum. If the bead fails to sink, a serum protein concentration of less than 6.0 per cent is indicated. However, we have found it impossible to purchase or prepare glass beads with specific gravities reasonably near to 1.0245 and have, therefore, devised the following simple method whereby hypoproteinemia can be determined with one drop of serum by a modification of the Hammerschlag method for determining the specific gravity of blood.

A test solution is prepared by transferring 500 c.c. of C.P. benzene to an amber-colored glass-stoppered bottle and adding 153.3 c.c. of C.P. chloroform. The specific gravity of the mixture is now adjusted to 1.0245 (determined with the Westphal Balance) by the addition of a few drops of either component. This should be carried out at a temperature of 15° to 20° C.

To perform the test, a small test tube is filled with the test solution and 1 drop of freshly obtained serum is allowed to drop on the surface of the liquid. If it sinks, or remains stationary near the middle of the tube, the serum contains 6.0 per cent or more of proteins. If the drop remains floating on the surface of the solution, the serum contains less than 6.0 per cent of proteins (equivalent to a specific gravity of 1.0245).

\*From the Department of Laboratories, the Israel Zion Hospital, Brooklyn.  
Received for publication, September 21, 1938.

This test was performed on the sera of 40 patients 24 of whom were clinically edematous, and confirmed by the determination of the total protein concentration and the serum albumin globulin ratio. Qualitative agreement was obtained in every case, as may be seen from Table I.

TABLE I

CASE NO	SERUM DROP	TOTAL SERUM PROTEIN PER CENT	ALBUMIN GLOBULIN RATIO
1	Floats	5.525	1.005
2	Floats	5.034	0.906
3	Sinks	7.085	1.786
4	Sinks	7.854	1.966
5	Sinks	7.119	1.665
6	Sinks	7.240	1.788
7	Sinks	8.022	1.894
8	Floats	5.500	1.250
9	Floats	5.612	1.222
10	Sinks	6.102	1.568
11	Sinks	6.118	1.442
12	Floats	5.990	1.446
13	Floats	5.724	1.102
14	Floats	5.446	0.812
15	Sinks	6.085	1.246
16	Sinks	6.115	1.346
17	Floats	5.712	0.912
18	Floats	5.704	1.006
19	Sinks	6.155	1.874
20	Floats	5.903	1.046
21	Sinks	7.904	1.800
22	Sinks	7.005	2.266
23	Sinks	6.268	2.044
24	Sinks	6.152	1.986
25	Sinks	6.246	1.788
26	Sinks	6.990	1.794
27	Floats	5.672	1.106
28	Floats	5.707	1.096
29	Floats	5.880	1.246
30	Sinks	6.680	1.990
31	Floats	5.912	0.990
32	Sinks	6.110	1.378
33	Sinks	6.464	1.788
34	Sinks	6.550	1.808
35	Sinks	7.256	2.246
36	Sinks	7.800	2.556
37	Floats	5.022	0.611
38	Sinks	6.660	1.798
39	Sinks	6.244	1.895
40	Floats	5.826	1.034

## REFERENCE

Bing, J. A Rapid Method for Determining Hypoproteinemia With a Glass Bead, *Acta med Scandinav* 94: 619, 1938.

## TWO NEW FILTERS, USING THE SEITZ E. K. STERILIZING PADS\*

AARON BROWN, M.D., NEW YORK, N. Y.

THOSE who are working in the field of applied immunology are often handicapped when they have very small quantities, i.e. (2 or 3 c.c.) to sterilize. To overcome this difficulty, two new types of bacteriologic filters will be found valuable in serologic laboratories.

The smallest Seitz filter now used is one with a capacity of 30 c.c. The standard filter pad is 35 mm. in diameter, and when saturated will absorb about 5 c.c. of fluid. With only 2 or 3 c.c. of human serum (for passive transfer) to be filtered, we cannot pass this amount through without diluting the serum.

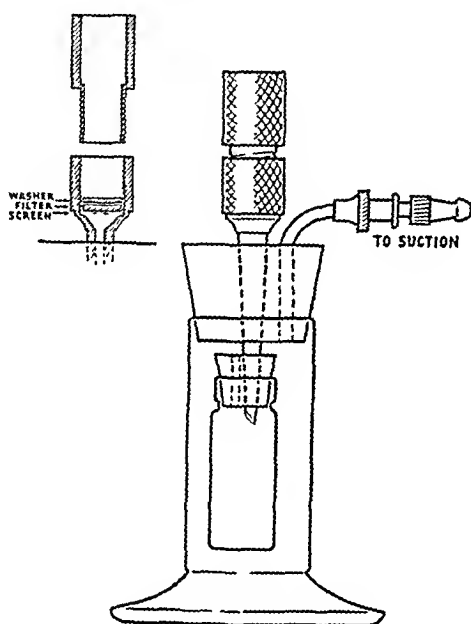


Fig. 1.

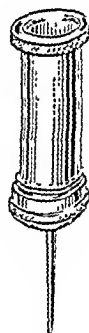


Fig. 2.

The microfilter apparatus illustrated in Fig. 1 meets our purpose. It consists of a modified small Seitz filter, having a capacity of only 3 c.c. It uses a small E. K. pad, 10 mm. in diameter, supported on a wire screen. The filter fits into a special glass jar, held in place by a rubber stopper. The filtrate vial is attached to the stem of the filter inside the jar and is capped with a sterile rubber stopper after filtration. The suction tube is in the large stopper. The entire apparatus is autoclaved as a unit.

A large filter (Fig. 2), with a capacity of 250 to 300 c.c., has been devised. This filter takes a regular No. 6 E. K. pad. The tops are interchangeable with a regular No. 6 Seitz screw type filter. This filter will be found useful when larger quantities are to be filtered, as with pollen extracts, stock dusts, diluting fluid, etc.

\*From the Department of Medicine, New York University, College of Medicine. We are grateful to the National Surgical Supply Co., New York, who have cooperated in devising and manufacturing both types of filters illustrated.

# A MANUAL OF NEUROHISTOLOGIC TECHNIQUE

OSCAR A. TURNER, M.D. NEW HAVEN, CONN.

(Continued from the May issue page 887)

## CHAPTER VIII

### CONNECTIVE TISSUE STAINS

Connective tissue stains are useful to the neuropathologist for the study of the proliferation of small vessels, the changes occurring in the walls of larger vessels, and particularly for the analysis of the constituent tissues of intracranial tumors. Of the various methods available the following have been selected because of the ease with which they are carried out and the generally reliable results they give.

*Mallory's Acid Fuchsin Aniline Blue Orange G Stain*—There are many modifications of this method, but the principle remains the same in all. The following is most commonly used and gives good results. Zenker fixed tissue, embedded either in paraffin or celloidin, is used. The results on formalin fixed tissue are inferior unless the sections have been treated with mordants prior to staining.

1 Stain sections in 0.5 per cent aqueous acid fuchsin for five minutes or longer, depending upon the freshness of the tissue.

2 Rinse in water. Extended washing will remove the dye from the tissue.

3 Treat in 1 per cent aqueous phosphomolybdic acid for thirty seconds to one minute.

4 Rinse rapidly in water.

5 Stain for two to twenty minutes in the following mixture:

Water soluble aniline blue	0.5 gm
Oxalic acid	2.0 gm
Orange G	2.5 gm
Water	100.0 cc

Heat to boiling, allow to cool, and filter.

6 Rinse rapidly in water. Differentiate and dehydrate in several changes of 96 per cent alcohol.

7 Complete dehydration in absolute alcohol. Clear in several changes of xylol and mount in balsam. For celloidin sections, use 96 per cent alcohol and clear by the blotting paper xylol method.

Collagen, reticulum, connective tissue amyloid, muens and certain other hyaline substances are stained blue. Nuclei, cytoplasm, fibroglia, myglia,

neuroglia, and fibrin assume a brilliant red color. Erythrocytes and myelin sheaths are yellow, while elastic fibers vary between a pale pink and yellow. The collagen and reticulum take the stain more sharply than do the fibrin and smooth and striate muscle fibers. Axis cylinders are stained red. To bring out the collagen fibrils as sharply as possible, the staining with acid fuchsin may be omitted. Then the nuclei and protoplasm stain yellow, and the blue reticulum and fibrillae are in sharp relief.

For formalin-fixed tissue, mordanting may be done with the Weigert mordants, as suggested by Kernohan.

*Haythorn's Modification* of Mallory's stain has been found to give good results. Paraffin sections of Zenker-fixed tissue, or formalin-fixed tissue which has been treated in the Weigert mordants, are used.

1. Stain sections for a half hour in alum or Boehmer's hematoxylin.

2. Rinse in water and mordant for two minutes in the following alum-orange G solution.

Dissolve 2.0 gm. of orange G in:

Alcohol 10 c.c.

Normal hydrochloric acid 1 c.c.

Water, sufficient to make 175 c.c.

When thoroughly dissolved, add:

Saturated iron alum solution, aqueous 75 c.c.

3. Place in tap water for five minutes.

4. Stain for three minutes in 0.5 per cent aqueous acid fuchsin.

5. Blot off the excess stain.

6. Stain for twenty minutes in the following modified aniline blue-orange G solution:

Aniline blue 2.5 gm.

Orange G 2.5 gm.

Saturated aqueous phosphomolybdic acid solution, to make 100.0 c.c.

Dissolve with the aid of heat.

7. Differentiate by the drop bottle method, using 95 per cent alcohol. Complete dehydration in absolute alcohol, clear in xylol, and mount in balsam.

The method gives results which are similar to those obtained by the original Mallory method. However, the nuclei are stained a deep black brown, and the erythrocytes take a deep orange-yellow color more constantly than in the original formula. In addition, there is an increased transparency to the sections which facilitates detailed examination of the tissue.

*Perdrau Silver Impregnation.*—This method is one of the best for the demonstration of connective tissue and is particularly suited to tumor mate-

nal Paraffin, celloidin, or frozen sections of formalin fixed tissue may be used. The method, essentially as given by Bailey and Miller, is as follows

1 Blocks of the formalin fixed tissue should be washed twelve to twenty four hours in distilled water previous to sectioning. Frozen sections should be cut at 15 to 25 microns. Paraffin sections should be brought to distilled water and allowed to remain there overnight, changing the wash water several times. Paraffin sections may be cut at 6, 8, or 10 microns.

2 Treat in 0.25 per cent potassium permanganate for twenty to thirty minutes. The 0.25 per cent solution should be prepared fresh from a 2 per cent stock solution.

3 Wash in distilled water.

4 Place in Pal's decolorizer and allow to remain until white. Pal's decolorizer is composed of equal parts of 1 per cent aqueous oxalic acid and 1 per cent aqueous potassium sulfite.

5 Wash for three to four hours in distilled water. The sections may be allowed to wash overnight.

6 Place in 2 per cent silver nitrate overnight. It is best to keep them in the dark.

7 Wash for two to three minutes in distilled water.

8 Treat for forty to sixty minutes in Bielschowsky's ammoniacal silver solution.

To 5 cc of 20% silver nitrate add 10 drops of 40% sodium hydroxide. Dissolve the resultant precipitate by the addition of strong ammonia, drop by drop, avoiding an excess. Dilute to 50 cc with distilled water and filter. The solution should be prepared fresh before use.

9 Wash quickly in distilled water.

10 Reduce in 20 per cent formalin prepared with tap water. Allow sections to remain in this reagent for ten to twenty minutes.

11 Wash in distilled water.

12 Tone in a 1:5000 solution of gold chloride until the sections are an even violet color. Wash in distilled water.

13 Fix for one minute in 5 per cent sodium thiosulfate. The tissue should assume an even gray color, and frozen sections should become quite pliable.

14 Wash thoroughly in distilled water, dehydrate in ascending alcohols, and clear in several changes of xylol. Mount in balsam.

With this technique the finer strands of connective tissue, reticulum, and elastic fibers are stained an intense black. The more compact masses of fibrous tissue may occasionally appear reddish black. Nuclei, axis cylinders, neurofibrils, and myelin sheaths are not stained. It may be necessary to cover the paraffin sections with gelatin to prevent them coming off the slides during



the prolonged silver bath. Tissue may be embedded in gelatin before cutting on the freezing microtome.

The Bielschowsky silver solution should be prepared just before use, since it does not keep. As in other methods where silver salts are used as staining agents, the glassware must be chemically clean. All glassware must be rinsed in distilled water before use and, except where otherwise indicated, all solutions should be prepared with distilled water.

*Wilder Method for Reticulum.*—This is a more rapid method which is applicable to tissue fixed in formalin or Zenker's fluid. Celloidin, paraffin, or frozen sections may be used. The method is best applied to tumor tissue.

1. Paraffin sections should be cut at 6 to 10 microns. Celloidin sections may vary from 4 to 30 microns and may be stained in dishes or mounted on slides and attached with thin celloidin before staining. Sections are hydrated and brought to distilled water.

2. Treat with 10 per cent phosphomolybdic acid for one minute.

3. Wash in tap water, then in distilled water.

4. Dip in 1 per cent aqueous solution of uranium nitrate (sodium free) for five seconds or less.

5. Wash in distilled water for ten to twenty seconds.

6. Treat in Foot's silver diamino-hydroxide for one minute.

To 5 c.c. of 10.2% silver nitrate, add strong ammonium hydroxide, drop by drop, until the precipitate which forms is just dissolved. Add 5 c.c. of 3.1% sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonia. Make up to 50 c.c. with distilled water. In an amber, well-stoppered bottle this keeps for several weeks.

7. Dip quickly in 95 per cent alcohol.

8. Reduce in the following solution for one minute:

40% neutral formalin	0.5 c.c.
1% uranium nitrate	1.5 c.c.
Distilled water	50.0 c.c.

The formalin should be neutralized with an excess of magnesium carbonate.

9. Wash in distilled water.

10. Tone for one minute in 1:500 gold chloride.

11. Rinse in distilled water and fix in 5 per cent sodium thio-sulfate for one to two minutes. Wash well in tap water.

12. Dehydrate in ascending alcohols, clear in xylol, and mount in balsam. If a counterstain is desired, hematoxylin and eosin or hematoxylin with van Gieson stain may be used. The hematoxylin must be blued in tap water, as ammonia will dissolve the silver.

The sections may be treated with 0.25 per cent potassium permanganate instead of 10 per cent phosphomolybdic acid. If this is done, it is necessary

to rinse the sections and place them for one minute in a mixture of 1 part of Merck's concentrated 34 per cent hydropionic acid and 3 parts of distilled water. The sections are then washed and carried on from the uraninm nitrate treatment as usual.

Reticulum and collagenous fibers are stained black. There is some impregnation of the cell nuclei, and occasionally the cells are outlined. Often the parenchymatous tissue takes the stain lightly so that, although cellular definition is not clear, there is enough impregnation to interfere with a clean-cut picture of the supporting tissues. If the silver solution is too old, uneven staining of the reticulum results. The method is useful for studying tumors arising in the central nervous system and elsewhere.

*Klarfeld-Achúcarro Tannin-Silver Method*—Although paraffin-embedded material cannot be used, this still remains one of the best methods for the demonstration of connective tissue in the nervous system. It is of particular value in the study of general neuropathologic material. The tissue is fixed in either formalin or 96 per cent alcohol, embedded in celloidin and sectioned at about 15 microns.

1. Place sections in 10 per cent formalin and allow to remain twelve to twenty-four hours. Sections may remain here several days.

2. Wash in water.

3. Place sections in a saturated (100 per cent) aqueous solution of tannin and allow to remain for two to three hours in the incubator at 50° C. in a closed dish. The time may be shortened to twenty to thirty minutes by very careful heating with a flame.

4. Wash in distilled water until the sections lose their transparency.

5. Place sections in silver nitrate solution until they turn a brownish-yellow color. They should be kept moving during this time.

Add concentrated ammonia, drop by drop, to 5 c.c. of 10% silver nitrate until the precipitate which is formed is redissolved. Add 5 to 10 additional drops of ammonia and then distilled water to make 20 c.c. For staining, add 15 drops of this solution to 20 c.c. of distilled water. Prepare fresh before use.

6. Transfer sections directly to 10 per cent formalin for five minutes. It is best to use two changes, transferring to the second as soon as the first solution becomes discolored. It is not necessary that the sections become very dark.

7. Wash in tap water and then in distilled water.

8. Differentiate in the following solution:

0.5% aqueous potassium ferricyanide	10 c.c.
96% alcohol	5 c.c.

Differentiate until the cortex is light yellow and transparent.

9. Wash in distilled water for a half hour.

10. Dehydrate in alcohols, clear in xylol, and mount in balsam.

The connective tissue appears black upon a light yellow or yellow background. Nerve cells, axis cylinders, myelin sheaths, and other elements of the nervous system are not stained. The background should appear clear and light. Occasionally with large sections the background appears darker in some parts than others. This is due to the difficulty in keeping large sections flat in the various solutions. Constant agitation during the silver impregnation and reduction will help prevent this uneven staining. Tissue which is very old or which was fixed long after death will show considerable granularity of the stained portions.

## CHAPTER IX

### PITUITARY AND PINEAL STAINS

Many methods have been devised with the object of selectively staining the various types of cells in the anterior lobe of the pituitary gland. Many of these are quite complicated and require special fixatives. The following methods have been found to give fairly reliable preparations in which the three types of cells in the anterior lobe can be readily distinguished.

*Sparks' Method.*—Although the best results are obtained on tissue fixed in Orth's fluid, formalin-fixed material has been found to give good results. Paraffin sections, cut at 6 to 8 microns, are brought to water and stained as follows:

1. Immerse in 0.25 per cent aqueous aniline blue for sixty to ninety seconds. The basophilic cells should be stained a deep blue, and the remainder of the tissue a very light blue.
2. Wash in tap water for about a half minute. Excessive washing will remove the dye from the tissue.
3. Immerse in Mayer's hematoxylin for ten minutes.
4. Wash in tap water two to three minutes.
5. Treat in van Gieson's mixture for sixty to ninety seconds.

Saturated aqueous solution of picric acid	100 c.c.
1% aqueous acid fuchsin	10 c.c.

6. Wash in tap water for one minute. Prolonged washing will remove the stain.

7. Transfer to 96 per cent alcohol for one minute.

8. Complete dehydration in absolute alcohol for two to three minutes. Clear in xylol and mount in balsam.

The nuclear chromatin is stained a purplish blue or reddish blue. The basophilic granules are stained a deep blue and can be differentiated from the granules of the acidophilic cells which are olive green in color. The cytoplasm of the chromophobe cells is a light grayish blue. Erythrocytes are a bright yellow and are not easily confused with other elements. The dense collagenous fibers are bright red, while the loose collagenous tissue may appear either red or blue. The capillaries take a sharp blue stain. The colloidal

in the anterior lobe of the gland takes a color which varies from light yellow to lilac, while that in the posterior lobe is a light blue. Preparations retain their color for years without fading.

Practice is necessary to obtain the optimum results. Excessive washing easily removes the dye from the tissue. Minor variations in the staining time will be found necessary, depending upon the staining properties of the solutions. Overstaining in van Gieson's mixture will result in a greenish-blue discoloration of the basophile. The results are good on human material but are of less value on animal tissue where the differentiation obtained is less distinct. Poor results were obtained on guinea pig and rat pituitaries. Some difficulty may be experienced at first in interpreting the final product, particularly by one who has been accustomed to examining preparations in which the basophilic cells are stained blue and the acidophilic cells red.

*Bailey's Ethyl Violet-Orange G Stain*—This method is the same as that employed for the staining of fibrillary neuroglia. However, the tissue must be fixed in Regaud's solution and kept in the dark the fixative being changed daily. The procedure is then carried out exactly as referred to above. With this method the acidophilic granules are stained a deep purple and the basophilic granules remain unstained. The background is orange. The nuclear stain varies in intensity.

This same method is also used for the demonstration of blepharoplasts in tumor tissue. These appear as purplish blue or purple intracytoplasmic granules or small rods on an orange background. Formalin-fixed material, if mordanted for some days in 3 per cent potassium bichromate, may give good results but is unreliable.

*Mallory's Acid Fuchsin-Aurine Blue Method*—Although this method is essentially a connective tissue stain, it has been found to be an excellent selective stain for the pituitary, particularly for routine laboratory work. Although it may be used without modification as described in the chapter on connective tissue stains, Crooke and Russell have found the following modification advantageous for the study of the cells in the anterior lobe of the pituitary:

1. Paraffin sections are brought to water and mordanted for twelve to eighteen hours in the following mixture

2.5% potassium bichromate	95 parts
Glacial acetic acid	5 parts

2. Wash in running water for two minutes.
3. Transfer to Lugol's iodine solution for three minutes or more.
4. Decolorize in 95 per cent alcohol for one hour or more.
5. Stain for fifteen minutes in a 1 per cent aqueous solution of acid fuchsin.
6. Wash in running tap water for thirty seconds to five minutes.
7. Rinse in distilled water.
8. Counterstain in Mallory's aurine blue mixture for twenty minutes.

9. Wash in running tap water for two to five minutes.
10. Differentiate in 95 per cent alcohol, controlling decolorization under the microscope. This may require from twenty seconds to five minutes.
11. Complete dehydration in absolute alcohol, clear in xylol, and mount in balsam.

This method can be used on formalin-fixed tissue. Preliminary nuclear staining with hematoxylin before treatment in acid fuchsin is advisable. Ehrlich's hematoxylin has been found to give good results, treating the sections for forty-five minutes followed by differentiation in acid alcohol and washing in tap water until blue.

The basophilic granules are stained a deep blue in contrast to the acidophilic granules which take a brilliant red stain. The chromophobe cells are a light bluish gray. The nuclei are purplish brown and the nucleoli a bright reddish orange. The supporting tissues stain as described in the original Mallory method.

*Safranine-Acid Violet Stain.*—Bailey has described the following method for the two types of granules in the anterior lobe of the pituitary. The tissue is fixed in Regaud's fluid, embedded in paraffin, and sectioned at 4 microns or less.

1. Treat in 0.25 per cent potassium permanganate solution for one minute.
2. Wash in distilled water.
3. Decolorize in 5 per cent oxalic acid for one minute or until colorless.
4. Wash well in distilled water.
5. Cover slides with *Babes' aniline-safranine* mixture and heat to steaming.

2% aniline water	100 c.c.
Safranine, water soluble	in excess

Saturated by heating in a flask. Allow solution to remain hot by placing flask in hot water at 60° to 80° C. Filter.

6. Wash in distilled water.
7. Add to the slide the following solution and allow to remain for ten minutes.

Saturated aqueous solution of acid violet	30 c.c.
Distilled water	70 c.c.

8. Wash quickly in distilled water.
9. Blot carefully and wash quickly in anhydrous acetone. Place in toluol for a few seconds.
10. Flood slide with pure oil of cloves and differentiate in a mixture of 3 parts of oil of cloves and 1 part of 96 per cent alcohol. Control decolorization under the microscope.

11 Flood slide with oil of cloves. Pass through two changes of toluol and mount in balsam.

The eosinophilic granules are stained a deep reddish violet and the basophilic granules a deep blue. The mitochondria do not stain.

*Hortega's Stain for Pinal Parenchyma*—The cellular elements of the pinal body have been divided into the neuroglial and parenchymatous types. The following method was devised by Hortega for the demonstration of the latter group and is used for the study of normal as well as pathologic tissue.

The material is fixed in 10 per cent formalin for at least two days, and frozen sections are cut as thin as possible.

1 Place the sections in the following mixture and allow them to remain until they become dark yellow.

2% silver nitrate	10 cc
Pure pyridine	3 drops

They may remain in this solution for twenty-four hours at room temperature or for a shorter time in the incubator. Heating the solution to 50° C for five to ten minutes hastens the staining.

2 Wash in distilled water containing 2 drops of pure pyridine.

3 Impregnate in the following mixture, heating until the sections become dark ochre.

Solution of silver carbonate	10 cc
Pure pyridine	3 drops

The silver carbonate is prepared as described in Penfield's combined method for microglia and oligodendroglia, except that the solution is diluted to 50 cc rather than 75 cc. It is best prepared fresh before use.

4 Wash in distilled water.

5 Reduce in 10 per cent formalin.

6 Tone in 1:500 gold chloride, warming slightly to intensify the color.

7 Fix in 5 per cent sodium hyposulfite solution.

8 Wash in distilled water. Mount sections on a glass slide from 50 per cent alcohol, blot firmly, complete dehydration in absolute alcohol, and clear in xylol. Mount in balsam.

The results are consistently good if the sections are cut thin. The neuroglial cells and fibers are not stained. The parenchymatous cells are colored a dark brown to brownish black, depending upon the depth of toning. They are characteristic large cells with many long processes which end in bulblike swellings. The latter are embedded in the interlobular and perivascular connective tissue. These swellings are deeply stained, often more so than the cell bodies. In thick sections the nuclei are relatively indistinct, but in thin prep-

arations they take the stain less deeply and can be seen to occupy a large portion of the cell body. The corpora arenacea (psammoma bodies) are black or a very dark brown.

## CHAPTER X

### SPIROCHETE STAINS

There often arises the necessity of demonstrating the presence of spirochetes, usually *Treponema pallidum*, in sections of nervous tissue. While there are many methods for staining these organisms, only a few are applicable in routine work in the general pathology laboratory. All require a precise technique and painstaking care for good results, regardless of the method employed. Absolute chemical cleanliness is imperative. All glassware must be rinsed previous to use, both in distilled and double distilled water. Glass hooks are used to handle and transfer the sections, and care is taken that wherever possible the instrument is rinsed in double distilled water before placing in a solution. Whenever filter paper is used, it should be of the finely woven type, free from paper lint. When dry glassware is required, air is used as a drying agent rather than cloths. Solutions and reagents should be measured exactly.

Spirochetes may be stained either in single sections or in tissue blocks. The latter method gives more precise results and is indicated where research work is being carried out. The methods of staining single sections, while more rapid than the block methods, are all prone to more or less artifact formation, but nevertheless give satisfactory results.

*Dieterle Method.*—This method gives clear preparations and can be used on any formalin-fixed tissue and with frozen, celloidin, or paraffin sections. The best results are obtained on frozen sections cut at 10 to 15 microns. If paraffin or celloidin sections are used, the embedding material must be removed before staining.

1. Transfer sections to pure pyridine for fifteen minutes.
2. Wash in double distilled water.
3. Place sections in a 1 per cent solution of sodium-free uranium nitrate made with 70 per cent alcohol. Allow to remain for a half hour in the incubator at 55° C.
4. Wash briefly in double distilled water.
5. Transfer through 96 per cent alcohol.
6. Place sections individually in a 10 per cent solution of gum mastic in absolute alcohol. Allow to remain long enough for thorough infiltration with the alcohol to take place, about thirty seconds.
7. Transfer through 96 per cent alcohol to double distilled water.
8. Leave sections for one to six hours in a 1 per cent aqueous solution of silver nitrate in the incubator at 55° C. Protect from the light.
9. Wash briefly in double distilled water.

## 10 Reduce for ten to fifteen minutes in the following solution

Hydroquinone	1.50 gm
Sodium sulfite	0.25 gm
Neutral formaldehyde, Merck 40%	10.00 cc
Acetone	10.00 cc
Pyridine	10.00 cc
Distilled water, to make	100.00 cc

Mix, dissolve, and then add 10 cc of a 10% absolute alcoholic solution of gum mastic, making the reducing solution "milky." This should be prepared fresh just before use.

## 11 Wash briefly in distilled water

12 Dissolve out the gum mastic and dehydrate the sections by transferring through 96 per cent alcohol and acetone. Clear in xylol and mount in Canada balsam.

The spirochetes are stained a deep brown to black against a golden brown background. The background should be clear and the nervous and interstitial elements of the tissue not stained.

The gum mastic solution is prepared by dissolving 10 gm of gum mastic in 100 cc of absolute alcohol. This takes about three days. It is filtered through a triple filter before use. The celloidin is first removed with acetone when celloidin sections are used. They are then placed in the pure pyridine and carried through as outlined previously. About 8 to 10 sections are used so that trial reductions can be started after about one hour in the silver bath. The sections are washed thoroughly after each reagent but after the silver bath a brief wash is sufficient to prevent overstaining.

*Nieto's Method*—This method also uses formalin fixed tissue. Frozen, paraffin, or celloidin sections may be used. The embedding agent must be removed from the embedded tissue before staining. Sections should be cut at 15 to 20 microns.

1 Place sections for ten minutes in pure pyridine.

2 Wash in three changes of double distilled water.

3 Place in a 1 per cent aqueous solution of mercuric nitrate for fifteen minutes in the incubator at 37° C.

4 Rinse in two changes of distilled water.

5 Transfer to a 0.2 per cent aqueous solution of silver nitrate. Heat carefully over a flame until bubbles just begin to form in the solution. Allow to remain about two minutes. Do not boil.

6 Transfer sections to a small dish containing 10 cc of 1 per cent aqueous silver nitrate solution. Four to five sections can be placed in here at the same time. Add 2 cc of a 5 per cent aqueous solution of tartaric acid, and finally, after mixing well, add 10 cc of a 1 per cent aqueous solution of pyrogallol acid. Cover the dish and allow sections to remain until they are yellowish brown. Agitate the sections in the reducing solution to obtain an even reduction.



7. Wash well in double distilled water.

8. Dehydrate in ascending alcohols, clear in xylol or carboxylol, and mount in balsam.

The spirochetes are stained a deep brown or brownish black upon a golden-yellow background in which none of the elements of the nervous tissue appear. Care should be taken that the sections are not overheated in the silver bath. Reduction begins immediately with the addition of the pyrogallie acid and should be carefully watched. The tartaric acid and the pyrogallie acid solutions may be mixed in proper proportions and added together. The time required in the developer varies with the individual tissues and often with the same tissue from time to time. Five minutes is usually sufficient for tissue from parietic brains. Reduction takes place rapidly in tissue from congenital syphilis, while sections from syphilitic aortitis are reduced relatively slowly. Sections of brain tissue are removed when the gray matter has taken a yellowish-brown color. The white matter usually appears darker than the gray.

Occasionally there is a slight deposit of silver on the surface of the sections. This often cannot be avoided, but does not detract from the usefulness of the method. The method is relatively rapid and can be completed in less than one hour. It can be applied to all types of tissues.

*Jahnel's Gold-Silver Method.*—This is Jahnel's latest method for single sections and is taken from Wertham. The method gives particularly clear preparations. Frozen sections are cut at 20 microns from material which has been well fixed in formalin.

1. Leave overnight in 96 per cent alcohol.
2. Wash briefly in distilled water.
3. Transfer to a 1 per cent aqueous solution of uranium nitrate and allow to remain for fifteen minutes in the incubator at 37° C.
4. Wash in two changes of distilled water.
5. Transfer sections to 1 per cent silver nitrate where they remain for twelve to twenty hours in the incubator.
6. Wash well in two changes of distilled water.
7. Transfer to the following mixture:

Distilled water	10 c.c.
1% aqueous gold chloride (Merck)	4 drops
1% uranyl chloride (Schering)	4 drops
Prepare fresh before use.	

Leave sections in this mixture in the incubator at 37° C. until the spirochetes are stained. This is usually one-half to one hour. Over-treatment in this is apt to cause the formation of precipitates. About fifteen to twenty minutes may be sufficient, but the time must be determined for the tissue being stained.

8. Wash thoroughly in distilled water.

9. Treat for five minutes in 5 per cent aqueous sodium thiosulfate.

10. Wash thoroughly in distilled water

11. Dehydrate in ascending alcohols, clear in xylol, and mount in balsam.

The background should be clear and the spirochetes stained distinctly. There is some impregnation of the elements of the tissue. It is important that the sections be protected from the light as much as possible up to the time they are fixed in the thiosulfate solution. Undue exposure to light before fixation causes a dark red or reddish-purple color.

*(To be continued )*

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M D, ABSTRACT EDITOR

## BRONCHIOGENIC CARCINOMA, Incidence of Primary, Matz, P. B. J A M A 111 2086, 1938.

The incidence of primary bronchiogenic carcinoma in the veterans' population has been increasing during the past few years. This increase is both absolute and relative.

The absolute increase of this type of tumor is unrelated to the increase of cancer in general. The factors which may be responsible for the absolute increase of cancer of the lung are the large percentage of preceding diseases of the respiratory tract and the fact that a comparatively large number of the persons in this group were in occupations which were accompanied by exposure to irritations of the respiratory tract and traumatizations of various kinds.

One of the factors causing a relative increased incidence of bronchiogenic carcinoma is the added interest in cancer on the part of the physicians of the Veterans Administration resulting in an increase of the number of cancers of the lung diagnosed ante mortem. In many of the cases in which carcinoma was suspected but was not definitely diagnosed ante mortem, post mortem examination was made, hence the increased incidence of this type of tumor in autopsy statistics.

Another factor which may be held responsible for the relative increase in the incidence of cancer of the lung is the entrance of the veterans' group into the cancer age period.

A comparative study of the data on the incidence of cancers of the lung according to age in the civilian population and in the veterans' group shows that the largest number of civilian patients are older than the veterans. One may, therefore, expect that as the veteran grows older an increasing number of primary bronchiogenic carcinomas will be seen among them.

## TRANSFUSIONS: A Method for Recognition of Blood Subgroups A<sub>1</sub> and A<sub>2</sub>, Davidsohn, I. J. A. M. A. 112: 713, 1939.

Clinical experience, as it is expressed in the literature, suggests (1) that the selection of a donor according to known methods does not assure the absence of blood transfusion reactions; (2) that unexpected reactions are not uncommon when a donor of the same blood group as that of the patients is employed, especially when the group is O or A<sub>1</sub>; (3) that reactions are particularly frequent when so called universal donors are employed.

Available serologic data suggest (1) that subgroups A<sub>1</sub> and A<sub>2</sub>, A<sub>1</sub>B and A<sub>2</sub>B are not always compatible; (2) that subgroups A<sub>1</sub> and A<sub>2</sub>B are not infrequently mistaken for B or of other groups, particularly for O and B; and (3) that some transfusion reactions, even fatal ones, are well explained by these circumstances.

The method presented offers two advantages. 1 The high titered, easily produced and highly specific rabbit immune serum permits prompt recognition of blood group A<sub>1</sub>, A<sub>2</sub>, AB, including the feebly agglutinating subgroups A<sub>1</sub> and A<sub>2</sub>B. 2 A proper dilution of the serum, as determined by titration, makes it possible to differentiate subgroup A from A<sub>1</sub> and A<sub>2</sub>B from A<sub>1</sub>B without delay. Both procedures can be carried out within a few minutes.

## UNDULANT FEVER: Histology of the Cutaneous Reaction to Brucella Melitensis Antigen, Gersh, I., and Black, W. C. Arch. Path. 27: 307, 1939.

The tissue reaction to the intradermal injection of *Br. melitensis* var. *abortus* antigen in 12 persons giving a positive reaction has been studied histologically. The reaction is characterized by a

inflammatory. When it is mild, it is characterized by infiltration of the derma by lymphocytes and monocytes, when it is severe, it is accompanied by connective tissue necrosis and infiltration by polymorphonuclear leucocytes.

**TUBERCLE BACILLI, Demonstration of by Culture and by Guinea Pig Inoculation, Guggenheim, A., and Finkelstein, M. Am Rev Tuberc 39 397, 1939**

Guinea pig inoculation was superior to culture as a means of demonstrating tubercle bacilli in all sorts of material.

With improvement in culture technique, the gap between these two methods of examination has decreased.

A few specimens negative by means of guinea pig inoculation were positive by culture.

An increase in the number of direct smears performed before resort to culture or guinea pig inoculation resulted in an increase in the percentage of specimens remaining negative after these more sensitive methods were used. This was true particularly of sputum, which was examined by direct smear much more frequently than other material. It would seem that a good many cultures and guinea pig inoculations might be made unnecessary if this practice were extended to urine, pleural draining sinuses and other materials easily available for repeated examination.

The best procedure for demonstrating tubercle bacilli in suspected material would seem to be (a) repeated direct smears, as frequently as circumstances permit, (b) one or more cultures, if smears are persistently negative, (c) guinea pig inoculation of all material negative by culture.

No case should be considered negative until all means of examination have repeatedly failed to demonstrate tubercle bacilli in the suspected material. The exact number of examinations necessary depends on the individual case.

**TRICHINOSIS, Blood Chemistry in Human, Pierce H B, Hartman, E, Sumcox, W J, Aitken, T, Meservey, A B, and Farnham W B. Am J Hyg 29 75, 1939**

Under the conditions of this study, namely during the second and third months after trichinosis infection in human beings, the following conclusions may be drawn:

Blood calcium, chloride, sugar, and nonprotein nitrogen were near the low normal levels early in the study.

The above constituents increased in amount during the period of convalescence.

Inorganic phosphate was slightly elevated throughout the study.

The calcium phosphorus ratios were depressed slightly in early periods due to a low calcium and moderately elevated inorganic phosphorus.

Values for cholesterol fell within the accepted range for normal human beings.

The addition of cod liver oil and calcium gluconate to the diet of human trichinosis patients had no marked effect upon any of the constituents determined.

**LEPROSY, Complement Fixation in, and Other Diseases by the Witebsky, Klingenstein and Kuhn Antigen, Lowe, J, and Greval, S D S. Indian J M Research 26 833, 1939, 26 843, 1939**

The antigen is essentially a solution of human tubercle bacilli in benzol after extraction with alcohol, pyridine, and acetone.

A technique of complement fixation for leprosy has been devised, using the hemolytic system of the method No. IV of the report of the (British) Medical Research Committee (now Council) on the Wassermann test, and W K K antigen.

The sensitiveness of the reaction in lepromatous type of the disease is of a very high order. Like a strongly positive Wassermann reaction of secondary syphilis, a positive reaction is not likely to be missed. The sensitiveness in neural leprosy is low.

The specificity of the reaction is not of a very high order. Kala azar gives a positive reaction. Doubtful reactions have sometimes been obtained in malaria, syphilis, dermal leishmaniasis, and tuberculosis. A Wassermann positive case has also given a + reaction.

The utility of the reaction is considerable in the diagnosis of the lepromatous type of the disease, but little in the diagnosis of the neural type. It may help in evaluating arrest of the disease in previously positive cases.

The procedure involved in putting up the test is brief, and the reagents used are standardized. A quantitative reading can be easily obtained.

The results of the test in 250 cases of leprosy, 46 cases of syphilis, 20 cases of leishmaniasis, 3 cases of tuberculosis of the lungs, and 43 cases of other diseases, are given.

Of 117 cases of leprosy of the lepromatous type, 98.3 per cent showed inhibition of hemolysis, 83.77 per cent showing complete inhibition.

Of 133 cases of neural leprosy, only 41 (31 per cent) showed some inhibition of hemolysis, only 12 of these showing complete inhibition. These 133 neural cases included 11 bacteriologically positive cases of whom all showed some fixation of complement, complete in 7 cases.

The results of the test in cases of leprosy are found to correspond very closely to the results of examination of the skin for *M. leprae*. Of cases in which bacilli were found, nearly all showed some inhibition of hemolysis in the test.

The test is considered to be of little value in diagnosis or prognosis, for inhibition of hemolysis is found only in cases in which diagnosis is easy, and the test fails to differentiate between the neural cases showing bacilli in smears which often have a good prognosis and the lepromatous cases which have a bad prognosis.

Of 46 cases of syphilis with a positive Wassermann test, 20 per cent showed inhibition of hemolysis, usually incomplete. Of 3 cases of tuberculosis of the lungs all showed partial inhibition of hemolysis. Of 20 cases of leishmaniasis, 18 showed complete inhibition of hemolysis. All 43 cases of other diseases gave negative results.

The test is, therefore, not specific for leprosy, since in other diseases, chiefly leishmaniasis, positive results can be obtained, and since in leprosy of the mild neural type negative results are usually obtained.

**GOLD SOL, Making, for Cerebrospinal Fluid Tests, Levine, B. S.** Ven. Dis. Inform. 20: 41, 1939.

The following formula is recommended for the preparation of 500 c.c. of gold sol:

Distilled water	460 c.c.
Gold chloride (1 per cent solution)	5 c.c.
Sodium citrate (1 per cent solution)	25 c.c.
Hydrogen peroxide (fresh 3 per cent solution)	5 M.
diluted in 10 c.c. of distilled water	

Heat the distilled water to brisk boiling. Remove from the hot plate. Add 5 c.c. of the 1 per cent gold chloride solution. Shake for a few seconds. Add 25 c.c. of the 1 per cent sodium citrate solution. Shake for ten or fifteen seconds. While shaking, add the 10 c.c. of distilled water which contains the 5 minims of hydrogen peroxide. Continue shaking for a few seconds. Let it rest on the table until the reaction is completed. This usually takes from one-half to one minute.

Multiples or proportional fractions of the above volumes can be used accordingly as more or less of the sol is needed. It was found that with this method there was no need to keep a large amount of the sol in stock. As little as 100 c.c. can be made with the same ease and with as high a degree of reliability as larger volumes. Experience has shown, however, that the reaction proceeds in a more regular manner in the larger volumes.

**SILICOSIS, Basophilic Erythrocytes in, Neal T A Am Rev Tuberc 38 629, 1939**

From several series of examinations of the blood of workers in nonlead dusty trades, and of hospital patients suffering from tuberculosis or silicosis with tuberculosis, no evidence was found to indicate that common industrial dusts other than lead or its compounds, provoke an increase in the usual number of red cells containing basophilic granules

**PREGNANCY, Relation Between Blood Plasma Proteins and Toxemias of, Dodge, E F, and Frost, T. T. J A M A 111 1898, 1938**

In pregnant women the blood plasma albumin is decreased below the level found in nonpregnant women. Toxemias of pregnancy uncomplicated by organic disease are associated with a significant decrease of plasma albumin and a questionable increase of the globulin. The increase of the globulin needs further investigation.

Pregnancy without signs of toxemia and a diet deficient in protein causes a decrease in plasma albumin.

An increased intake of protein in cases of mild toxemia causes an alleviation of objective and subjective symptoms and signs and is well tolerated by the patient.

**STREPTOCOCCUS, Hemolytic, Cultural Characteristics of Potentially Virulent Strains Ward, H K, and Rudd, G V Australian J Exper Biol & M Sc 16 180, 1938**

If hemolytic streptococci from human sources are grown in two media (a) serum broth and (b) serum peptone with just sufficient salt to suspend the developing colonies, the potentially pathogenic group A (Lane-Field) strains with very few exceptions, can be differentiated by their growth characteristics from hemolytic streptococci belonging to the other serologic groups.

Capsulated group A strains grow diffusely in the serum broth and the colonies in the serum peptone agar have a feathery semitransparent appearance.

Noncapsulated group A strains grow flocculently in serum broth, with a marked opalescence in the supernatant fluid. The colonies in the serum peptone agar are compact and opaque.

Hemolytic streptococci from human sources, not belonging to group A, grow flocculently in serum broth without opalescence in the supernatant fluid, and the colonies in serum peptone agar are compact and opaque.

Occasionally a group A strain grows in an atypical manner in the two special media and may be mistaken for a hemolytic streptococcus not belonging to group A. Such atypical strains are unstable and throw off characteristic capsulated variants on cultivation.

The opalescence in serum broth produced by all the noncapsulated and a few of the capsulated group A strains appears to be due to a ferment which denatures the serum protein.

It is suggested that the feathery colony in serum peptone agar which is characteristic of the capsulated group A strains is due to the strong negative charge on the surface of the developing organism, resulting in the mutual repulsion of the chains of young cocci as they grow out into the medium. That this phenomenon is not necessarily associated with capsulation is indicated by the fact that nonmotile virulent typhoid bacilli also develop feathery colonies.

**TRICHOPHYTIN TEST, Value of, Lewis, G M, MacKee, G M, and Hopper, M E Arch D & S 38 713, 1938**

From a consideration of the varied responses to trichophytin, to oidiomycin, and to a bacterial vaccine, together with observation of a high percentage of positive reactions in patients with certain fungous eruptions and of a high percentage of negative reactions in patients without evidence of fungi on culture, the authors believe that the reaction to the trichophytin test is specific.

The specificity is considered to be chiefly determined by genus rather than by species.

Fungi of different species vary greatly in their ability to sensitize. In some instances patients with severe mycosis may show a negative reaction to trichophytin.

The reaction to trichophytin is always positive when the subject has a dermatophytosis.

There is no difference in the response to trichophytin in patients with dermatophytosis and in patients without "id" but with an inflammatory eruption due to the same organism.

The repeated intracutaneous injection of trichophytin into a test site will not cause a false positive reaction.

Some patients are sensitive to very dilute solutions of trichophytin, and others fail to react to solutions of concentrated trichophytin. Most patients show a gradual increase in the response according to the strength of solution.

The patch test with trichophytin is not as delicate or as reliable as the intracutaneous test.

Little difference was noted in the size of the response when different parts of the body were tested. There was usually a slight increase in the size of the reaction when a site near a fungous focus was used.

The immediate wheal reaction occasionally occurs and may be associated with circulating antibodies, demonstrated by passive transfer tests.

The test should be an integral part of the examination of a patient for suspected fungous disease. A positive reaction is not in itself diagnostic; when it agrees with other findings, confirmation is obtained; the prognosis is hopeful, and treatment should be conservative. If the test gives a negative result in the presence of proved fungous eruption, the immediate prognosis is poor, and treatment should be vigorous and sustained. If the test shows a negative reaction when the fungous examination gives a negative result and the primary eruption is inflammatory, one is justified in calling the rash nonmycotic. The authors think this is especially important when the eruption is eczematous, and particularly when it involves the hands, because the diagnosis of such eruptions is frequently a matter of dispute in compensation work.

#### BLOOD REGENERATION, Observations on, Schidt, E. *Am. J. M. Sc.* 196: 632, 1935.

After a hemorrhage, even if production of red blood cells is not increased, one may expect a rise in percentage of reticulocytes as the same (normal) number of reticulocytes will give a higher percentage when the erythrocyte level is lowered.

In the literature, the assumption of a high reticulocyte percentage (up to 25 per cent) after hemorrhage has been a dominating concept.

In 17 patients given iron and in 10 patients not so treated, but all given the same standard diet, the reticulocytosis very rarely exceeded 10 per cent.

As a contrast to these findings, 6 patients with a low color index on admission gave values up to 28 per cent. These patients are supposed to have had an iron deficiency anemia before the hemorrhage.

The reticulocytosis in patients given iron did not greatly exceed that in patients not so treated.

A correlation was found between the height of reticulocytosis and the lowest erythrocytic level.

In individual cases, the size of the reticulocytosis did not give any indication as to the regeneration rate.

# *The Journal of Laboratory and Clinical Medicine*

VOL 24

JULY 1939

No 10

## CLINICAL AND EXPERIMENTAL

### HEMATOLOGIC STUDIES IN ACUTE INFECTIONS\*

E L AMIDON, M D, BURLINGTON, VT

THE value of the white and differential counts in acute infections is accepted generally, as is also the "shift" as determined by Schilling. Some observers<sup>1, 4</sup> have noted valuable informative evidence as shown by the qualitative changes in the neutrophils. The purpose of this work is to determine the relative value of these different methods.

Several methods of examination were made on the same blood, and the information thus gained was evaluated and compared. These methods were (1) total white count, (2) differential count, (3) estimation of the percentage of young neutrophils, (4) estimation of the percentage of neutrophils containing "toxic granules", (5) estimation of the percentage of neutrophils showing evidence of severe damage, (6) calculation of the "index of resistance".

The percentage of young neutrophils, the so called "shift," was determined by the method of Farley<sup>5</sup>. The count is reported as the percentage of non filamented neutrophils per hundred leucocytes.

Probably Cesaris Demil<sup>6</sup> was the first to describe the large, blue staining, peroxidase negative granules<sup>7</sup> in the cytoplasm of the neutrophils. These are the "toxic granules" of Hirschfeld, Tuerk, Nageli, and many others. The result is reported as the percentage of neutrophils containing such granules.

Living<sup>8</sup> in 1901 called attention to the evident degenerative changes sometimes seen in severe toxemias. Bodkin<sup>9</sup> in 1892 expressed the importance of such changes. Strumia<sup>10</sup> has chosen to separate the degenerated neutrophils into three groups: (1) those cells showing only "toxic granules", (2) those cells showing clumping of the cytoplasmic granules with vacuolization, (3) those

\*From the Departments of Medicine and Pathology, University of Vermont College of Medicine.

Summary of thesis submitted as partial requirement for degree of M S in Medicine from the Graduate School of Medicine of the University of Pennsylvania.

Received for publication December 19 1938



cells showing "coagulation necrosis" and edema, terminating in nuclear rupture. The cells classed as "degenerated" neutrophils were those listed in Strumia's third group. The result is reported as the percentage of neutrophils showing these degenerated changes.

The "index of resistance," as suggested by Wilson,<sup>11</sup> was calculated from the formula  $(T-10)-(P-70)$ , in which T is the total white count in thousands and P is the percentage of neutrophils. It apparently is of little significance when the white count is less than 10,000.

The above studies were done on fresh blood smears made on slides and immediately stained following the technique recommended by Strumia.<sup>10</sup>

The first studies were made on cases of acute appendicitis, counts being done on admission, and on the third and sixth hospital days. The interpretation of each count was compared then to the clinical course of the patient and the pathologic report of the appendix. Following these studies, other acute infections were studied in a similar manner. A total of 330 patients were studied and 550 examinations were made.

In order to compare these different examinations it was necessary to reduce the findings to common terms. Mendell<sup>12</sup> has established the numerical limits for the nonfilamented and "toxic-granular" count which seems acceptable for our use here. The interpretation of the lesion, as indicated by the percentage of degenerated cells, is our own choosing and may differ from that of other observers.

	NONFILAMENTED	"TOXIC GRANULES"	DEGENERATED
Mild infection	-16	-20	-10
Moderate infection	17-30	21-40	11-25
Severe infection	31-40	41-70	26-40
Grave infection	41 up	70 up	41 up

Fourteen of the patients studied had diagnoses of acute appendicitis, from whom gangrenous appendices were removed. These cases are listed in Table I with the blood counts, the interpretation, the pathologic diagnosis, and a few clinical notes.

TABLE I  
GANGRENOUS APPENDICITIS

PATIENT	PREOPERATIVE			THIRD DAY			SIXTH DAY			COMMENT
	NF	"T.G."	Deg. I-R	NF	"T.G."	Deg. I-R	NF	"T.G."	Deg. I-R	
1	G	G	G - 9	G	G	S - 5	G	G	S - 2	Pelvic abscess
2	G	S	M - 6	S	M	Mi 0	G	G	Mi *	
3	M	S	M - 5	S	G	M - 2	S	S	M *	
4	G	Mi	Mi -15	G	G	N *	G	G	N *	General peritonitis
5	M	Mi	N 0	G	S	N 1	G	G	N - 5	General peritonitis
6	G	Mi	M -18	G	S	M *	S	S	Mi -16	General peritonitis
7	S	S	Mi -10	M	S	N 5	M	G	Mi *	Local peritonitis
8	M	M	M - 9	M	G	Mi -12	M	M	Mi - 7	Local Peritonitis
9	G	G	Mi 3	G	G	N - 3	G	G	Mi 0	General peritonitis
10	G	S	N -16	G	S	Mi -15	S	S	Mi 0	
11	G	M	G (Lived only few hours after admission.							
12	S	M	Mi - 4	S	M	Mi *	S	G	M 0	
13	G	G	S *	G	G	S *	G	G	G *	General peritonitis
14	G	G	M 0	G	S	M 1	G	Mi	Mi 4	

NF = nonfilamented, "T.G." = "toxic granules," Deg. = degenerated, I-R = index of resistance.

G = grave, S = severe, M = moderate, Mi = mild, N = normal, \* = white count below 10,000.

*Comment*—The percentage of nonfilamented cells indicated the presence of an infection in all of the 14 cases, and in all roughly paralleled the severity of the process. The percentage of "toxic granules" failed to show such a process in 4 of the patients, and the percentage of degenerated cells was not abnormally elevated in 7. Where applicable, the 'index of resistance' followed the clinical course closely.

From the foregoing it would appear that the nonfilamented cell percentage was the most helpful, while the percentage of degenerated cells was of the least value.

TABLE II  
ACUTE APPENDICITIS NOT GANGRENOUS

PATIENT	PREOPERATIVE				THIRD DAY				SIXTH DAY				COMMENT
	NF	"TG"	Deg	IR	NF	"TG"	Deg	IR	NF	"TG"	Deg	IR	
1	M	N	M <sub>1</sub>	*	M	S	M <sub>1</sub>	*	M	S	M <sub>1</sub>	*	Local peritonitis
2	G	S	M	-4	M	M	M <sub>1</sub>	*	M	G	M <sub>1</sub>	*	
3	S	S	M <sub>2</sub>	-3	M	M	M <sub>1</sub>	1	M	S	N	*	
4	M <sub>1</sub>	M	N	*	M	S	N	*	M	M	M <sub>1</sub>	*	Phlebitis third day
5	M <sub>1</sub>	M	M <sub>1</sub>	*	S	M	N	*	S	M <sub>1</sub>	N	*	
6	N	M <sub>1</sub>	N	*	M	M	M <sub>1</sub>	*	M	S	N	*	
7	G	N	N	*	S	S	N	*	S	S	M <sub>1</sub>	*	
8	M	M	M <sub>1</sub>	*	M <sub>1</sub>	S	N	*	M	M <sub>1</sub>	N	*	

*Comment*—In all of the 8 patients with acute appendicitis enumerated in Table II, the presence of an inflammatory process was indicated by the percentage of nonfilamented cells. The percentage of "toxic granules" indicated such a process in 6 of the patients. The percentage of degenerated cells was not sufficiently elevated to indicate any inflammation in 5 of the 8 patients.

In the group of 22 patients with appendicitis 14 of which were gangrenous and 8 of which were mildly acute the percentage of nonfilamented cells was elevated in 100 per cent, the percentage of "toxic granules" was elevated in 73 per cent, while the percentage of recognizable degenerated neutrophils was elevated in 43 per cent.

TABLE III  
PYELONIA

PATIENT	NONFILAMENTED				"TOXIC GRANULES"				DEGENERATED				INDEX RESISTANCE*	COMMENT
	M <sub>1</sub>	Mo	S	G	M <sub>1</sub>	Mo	S	G	M <sub>1</sub>	Mo	S	G		
1				1				1		1				Died following day
2				3				3		1	2		- 6, -10	Died in 3 days
3		1	2					2		3			0, - 3	Discharged 15th day
4		3	2	2			3	4		5	2		- 5, 10	Discharged 20th day
5				3				3		1		2	16, -24	Died in 4 days
6		2	2	3				7	1	5	1		- 7, 13	Discharged 15th day
7				6			2	4			2	4	-27,	Died on 8th day
8				5				5			4	1	- 4, -20	Discharged 41st day
9				1				1				1	- 3	Died day admitted
10		1		6			3	4		3	2	2	- 4, -15	Died on 7th day

\*Extreme limits

*Comment.*—In the 10 patients with pneumonia, 6 cases of which were fatal, the percentage of “toxic granules” indicated a grave process in all. The percentage of nonfilamented cells indicated a grave process in all of the 6 fatal cases, and one of moderate degree in all but one of the nonfatal cases. In much the same manner the percentage of degenerated cells indicated a grave process in 3 of the 5 fatal cases, and indicated a grave process in one of the 4 nonfatal cases.

It would thus seem that the percentage of nonfilamented cells was the most helpful and accurate index in this series, followed very closely by the percentage of degenerated cells. The percentage of “toxic granules” was of little help. Mention should be made of the fact that patient No. 8, who received a grave prognosis from both the nonfilamented and degenerated cells, received adequate dosage of sulfanilamide, and seemed clinically to be improved by it.

TABLE IV  
MISCELLANEOUS INFECTIONS

PATIENT	NONFILAMENTED			“TOXIC GRANULES”			DEGENERATED			INDEX RESISTANCE*	DIAGNOSIS	COMMENT
	Mo	S	G	Mo	S	G	Mo	S	G			
1			2			2			2	-10, -18	Typhoid	Died on 4th day
2		1	2			3	1		2		Typhoid	Discharged 31st day
3			2	1		1	1	1		-12, -19	Streptococcic bacteremia	Died on 4th day
4	1	3	3	6	2	1	9	1		-1, 12	Meningitis (pneumococcic)	Discharged 6th week
5			1			1			1	-4	Meningitis (pneumococcic)	Died day of admission
6			3			3	1	2		-1, -18	Erysipelas	Died on 4th day
7			2			2	2			-6, -14	Bacterial endocarditis	Died on 6th day
8			2			2			2	-4, -3	Gangrenous stomatitis	Died day of admission
9			2			2	2			-12, -13	Ruptured ulcer (peritonitis)	Died 4 days later
10	2	1			1	2				-7, 0	Acute cholecystitis	Discharged postop. 31st day
11		1	1		2		1	1		-8	Pulmonary tuberculosis	Discharged 2 wk. later
12		1	1		2			2		-5, 0	Meningitis (TB)	Died day following admission
13	1					1				2	Meningitis (TB)	Died day following admission
14		2				2	2			-5, 0	Pyelonephritis	Discharged recovered
15		1		1			1				Hydronephrosis	Died day of admission
16	Mild	3		1	1		Mild	3		-18	Pregnancy (appendicitis?)	Discharged 9th day
17	2			1	1		1	1		-11, -5	Appendicitis?	Discharged 10th day
18	1	2	20		2	16	2	3	13	-25, 20	Hemolytic streptococcus wound infection	Discharged 10th wk.
19		1	3		2	2	2	1		-7, -4	Appendicitis?	Discharged 5th day
20	1	1	1			3	Normal	3		-9, -7	Tonsillitis Salpingitis	Salpingectomy—eventful recovery
21		1	3		1	3	3	1		-9, 6	Staphylococcic bacteremia	Discharged 3½ months later

\*Extreme limits.

*Comment*—In the 21 cases of miscellaneous infections in Table IV, the information derived from each count is compared with the clinical findings in relation to the type and the severity of the process, together with the outcome. The count most closely corresponding to the clinical evidence, in our judgement, is listed as number 1, the other counts being graded 2 and 3 in the order in which they approach the first. The results are tabulated as follows:

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Nonfilamented	1	1	1	3	1	1	1	1	1	1	2	1	2	2	1	1	1	1	2	2	1
"Toxic granules"	1	3	2	2	1	1	1	1	1	2	1	2	1	3	2	2	2	1	2	1	1
Degenerated	3	2	3	1	1	1	2	1	2	3	2	2	1	2	1	1	1	1	3	1	1

It may be seen from the above that the percentage of nonfilamented cells indicated a process closely corresponding to our impression of the case clinically in 15 out of 21 cases (71 per cent). In the case of one patient, the conclusions derived from the percentage of nonfilamented cells were considered less accurate than those of the other counts. The percentage of "toxic granules" seemed to reflect the clinical condition accurately in 11 of the 21 cases (52 per cent). The conclusions derived from the percentage of degenerated cells seemed to be accurate in 11 of the 21 cases (52 per cent). In 6 of the listed cases the percentage of degenerated cells indicated a process which was considered the least accurate of the counts. In 6 cases the white count was not sufficiently elevated to calculate the "index of resistance," in 9 it seemed to follow the clinical course closely, and in 6 it was of questionable significance.

#### DISCUSSION

In estimating the value of these several methods of examination, the information gained must be balanced against the time effort and equipment required. The determination of the percentage of nonfilamented cells is relatively easy, requiring a small amount of time, no special technical skill, and appears to be subject to little individual variation. The counting of "toxic granules" is also relatively easy, requiring somewhat better staining, but again subject to little variation in individual examiners. The determination of the percentage of degenerated cells, on the other hand, is difficult. It requires good smears, a good stain, and very close examination of the neutrophils, necessitating the expenditure of more time. With the best obtainable technique the individual variations must be large. It would appear that only in exceptional circumstances could hospitals incorporate this procedure into the routine laboratory examinations. Probably more skill and experience would produce better results but from our experiences there would seem to be little reason for encouraging the adoption of such a procedure.

#### SUMMARY

In 22 cases of acute appendicitis, the percentage of nonfilamented cells was found to give more accurate information regarding the presence and severity of the process than the percentage of either "toxic granules" or degenerated cells.



# THE ANTACID PROPERTIES OF MAGNESIUM TRISILICATE IN NORMAL SUBJECTS AND PATIENTS WITH PEPTIC ULCERS\*

PHILIP KURTZ, M.D. INDIANAPOLIS, IND.

MAGNESIUM trisilicate has recently received attention as a means of neutralizing excess gastric acidity (Mutch<sup>1,2</sup>). In the presence of hydrochloric acid a colloidal gel is formed with a neutralizing power, which is partly chemical and partly adsorptive. The initial phase of neutralization is rapid and the subsequent phase prolonged. The toxicity is low and danger of alkalosis is minimized by the fact that magnesium trisilicate is insoluble in distilled water and weak alkalies. An excess is, therefore, not absorbed and regardless of the amount given, the gastric reaction will not go far below neutrality. This is a property which may be of clinical importance.

Mutch states that it does not disturb bowel motility and that it has a selective adsorbing power for certain bacterial toxins: putrefactive amines, and food poisons. Mann<sup>3</sup> reported a nine hour fractional test meal on a patient with a gastric ulcer receiving 1 dram of magnesium trisilicate every hour. Free hydrochloric acid was absent in 17 of 19 samples. Mutch reported 7 normal subjects with fractional gastric analyses following a standard meal with and without 35 grains of magnesium trisilicate. He demonstrated that a substantial reduction in hydrochloric acid occurred.

Since there is often variation in the results of consecutive gastric analyses on normal persons following Ewald or alcohol meals it was decided to test the antacid properties of magnesium trisilicate after administration of histamine acid phosphate, given subcutaneously. There is a question whether histamine stimulates the production of gastric enzymes<sup>4</sup> but it is known to be a powerful acid secretagogue. Therefore, it seems adequate as a test meal for demonstrating antacid properties of magnesium trisilicate.

## METHODS

Fourteen of the subjects were normal young adult white males, 21 to 33 years of age. Three of the subjects were white males, 23 to 45 years of age, with peptic ulcers previously demonstrated by x-ray. All 3 had typical symptoms and had not been treated for several days.

The subjects were in the postabsorptive state. A Rehfuess tube was passed and the fasting gastric contents were completely aspirated. Then they received 0.5 mg. histamine subcutaneously. This was not more than five minutes before the administration of water or water and magnesium trisilicate. On the control day, 50 c.c. tap water were administered by the Rehfuess tube, and on the

\*From the Indianapolis City Hospital, Indianapolis.

Received for publication August 2, 1938.

Magnesium trisilicate as Trisomin 1000 was kindly furnished by Eli Lilly and Co., Indianapolis.

test day, 50 c.c. tap water plus 15 grains magnesium trisilicate were given. Ten cubic centimeters of gastric juice were withdrawn three times at thirty-minute intervals following the histamine injection.

The control day preceded the test day in 9 normal cases and all 3 of the ulcers. The control day followed the test day in 5 of the normal cases.

TABLE I

THE AVERAGE TITRATABLE ACID AND PH OF GASTRIC JUICE OF 14 NORMAL SUBJECTS AND 3 PATIENTS WITH PEPTIC ULCER, USING HISTAMINE STIMULATION WITH AND WITHOUT ADMINISTRATION OF 15 GRAINS OF MAGNESIUM TRISILICATE

	TÖPFER'S REAGENT		PHENOL RED		pH	
	WATER	WATER + MAG. TRIS.	WATER	WATER + MAG. TRIS.	WATER	WATER + MAG. TRIS.
<i>Normal Subjects</i>						
	<i>acid per cent</i>		<i>acid per cent</i>			
Fasting	10.2	13.1	15.1	20.2	4.16	3.84
30'	37.6	9.4	42.3	14.3	1.94	3.24
60'	63.5	26.9	69.2	33.5	1.26	1.90
90'	52.3	22.3	61.3	31.6	1.36	2.29
<i>Patients With Peptic Ulcer</i>						
	<i>acid per cent</i>		<i>acid per cent</i>			
Fasting	36.3	19.2	47.0	29.5	1.63	2.03
30'	52.6	22.6	57.2	27.2	1.23	2.43
60'	92.3	50.3	96.5	54.3	1.13	1.40
90'	74.3	30.0	Av. of 2 98.5	39.7	1.13	1.66

Determination of pH by the colorimetric method of Brown<sup>6</sup> and titration of 1 c.c. of filtered gastric juice to pH 7.0 with 0.01 N sodium hydroxide, using phenol red as the indicator as recommended by Helmer and Fouts,<sup>7</sup> were done on each sample without delay. Titration of 1 c.c. filtered gastric juice with 0.01 N sodium hydroxide, using Töpfer's reagent as the indicator, was also done for comparison. The titration figures were multiplied by 10 to give the number of cubic centimeters of 0.1 N hydrochloric acid per 100 c.c. gastric juice or units of acid per cent.

#### RESULTS

Table I shows that 15 grains of magnesium trisilicate reduced the average titratable acidity of the gastric juice of 14 normal subjects 28.0 acid per cent at the thirty-minute interval, 35.7 acid per cent at sixty minutes, and 29.7 acid per cent at ninety minutes. This checks well with the decrease noted when using Töpfer's reagent as the indicator: 28.2 acid per cent at thirty minutes, 36.6 acid per cent at sixty minutes, and 30.0 acid per cent at ninety minutes. The pH was correspondingly increased.

A similar reduction in titratable acidity was found in 3 cases of peptic ulcer (Table I).

The ninety-minute samples of each person tested showed a reduction in acidity (the average decrease of all 17 at ninety minutes was 32.5 acid per cent) despite the fact that an appreciable amount of magnesium trisilicate was neces-

sarily removed with the thirty and sixty minute samples of gastric juice with drawn for analysis. It made no difference whether the control day preceded or followed the test day.

### CONCLUSIONS

The efficiency of magnesium trisilicate as a gastric antacid has been demonstrated following histamine stimulation in 14 normal subjects and 3 patients with peptic ulcer.

Reduction in free acid was still marked ninety minutes after administration of 15 grams of magnesium trisilicate.

### REFERENCES

- 1 Mutch, N. Silicates of Magnesium Brit M J 1 143 1936
- 2 Mutch, N. Synthetic Magnesium Trisilicate Its Action in Alimentary Tract, Brit M J 1 205, 1936
- 3 Mutch, N. Hydrated Magnesium Trisilicate in Peptic Ulceration Brit M J 1 204, 1936
- 4 Mann, W. N. Experiments on Neutralization of Hydrochloric Acid by Magnesium Trisilicate, Guy's Hosp Rep 87 101, 19
- 5 Toby, C. G. Does Histamine Stimulate Secretory Activity of Peptic Cells in Man? Am J Digest Dis & Nutrition 3 902 19
- 6 Brown, J. H. Colorimetric Determination of Hydrogen Ion Concentration of Small Amounts of Fluid, J LAB & Clin Med 9 91-4
- 7 Helmer, O. M., and Fouts, P. J. Gastric Acid Methods Am J Clin Path 7 41, 1937

## EFFECT ON BACTERIOPHAGE OF PRONTILIN PRONTOSIL, SULFAPYRIDINE AND OTHER ANTISEPTICS AND DYES USED IN SURGICAL PRACTICE\*

HELEN ZAITZEFF JERN, M.D. AND FRANK L. MELENEY, M.D.  
NEW YORK, N. Y.

### INTRODUCTION

**S**URGICAL infections are frequently caused by two or more pathogenic organisms each requiring a different method of attack. For the staphylococcus, the most common causative organism of wound infection, potent bacteriophages have been successfully employed in recent years<sup>1-30</sup>. The colon bacillus and *B. pyocyaneus*, which are commonly found in postoperative cystitis and in wounds infected by intestinal organisms, often promptly disappear under potent bacteriophage treatment. Nevertheless many wounds are infected with organisms for which no potent bacteriophages have yet been found. Among these are the large groups of streptococci. The hemolytic streptococcus which is often found in combination with *Staphylococcus aureus* has heretofore been an infective agent causing great concern to the surgeons. But recently sulfanilamide<sup>31-40</sup> and zinc peroxide<sup>41-4</sup> have been shown to be effective agents against it, the

\*From the Bacteriological Research Laboratory, the Department of Surgery, Columbia University, New York.

Received for publication September 6 1935



former as an internal medicament and the latter as a local applicant. However, it has been found that these chemicals have little or no effect on staphylococci and certain other pathogenic bacteria. The question arises whether they, as well as other specific or general antiseptics, can be used in combination with bacteriophage to combat mixed infections. The present paper attempts to answer this question.

#### LITERATURE

There is a long list of reports on the effect of antiseptics on bacteriophage. The literature on this subject is too voluminous to review here but reference is given in the bibliography.<sup>1-36</sup> Suffice it to say that these reports have shown that antiseptics in general inhibit or destroy bacteriophage. Some authors have shown that there is a differential action of certain antiseptics on different types and on different races of phage within the same type.

#### EXPERIMENTAL

Our study was undertaken for the purpose of determining whether certain antiseptics could be used with our phages in the treatment of mixed infections.

The antiseptics and dyes frequently employed on the surgical service of the Presbyterian Hospital, New York, are (1) prontosil (sulfanilamide); (2) 2.5 per cent prontosil; (3) Dakin's solution; (4) 2 per cent and 4 per cent boric acid; (5) red wash (1 per cent zinc sulfate); (6) 5 per cent aluminum acetate; (7) 95 per cent, 10 per cent, 5 per cent, and 0.5 per cent carbolic acid; (8) 1:10,000 silver nitrate; (9) 10 per cent argyrol; (10) 1 per cent acetic acid; (11) 25 and 70 per cent alcohol; (12) 2 per cent mercurchrome; (13) 1:1,000 and 1:10,000 merthiolate; (14) 1:500 and 1:5,000 metaphen; (15) 3.5 per cent iodine; (16) 1:500 azoehloramide; (17) 1:1,000 hexylresoreinol; (18) 1 per cent to 10 per cent zinc peroxide; (19) 1:2,000 methylene blue; (20) 2 per cent gentian violet. The surgical solutions include (21) physiological saline (0.5 per cent), 1 per cent and 5 per cent saline; (22) Ringer's solution; (23) 5 per cent dextrose solution in distilled water and 5 per cent dextrose in Ringer's solution; (24) 1 per cent novocain. The recent discovery of sulfapyridine (derivative of sulfanilamide) and its wide use in infections caused by a variety of pathogenic microorganisms made it necessary to carry through an additional series of experiments on the effect of this substance upon the action of bacteriophage.

All of our experiments were carried out with our staphylococcus, colon bacillus, and *B. pyocyaneus* bacteriophages and their corresponding bacteria. We employed two staphylococcus phages: (A) polyvalent phage "BK" of Gratia and (B) stock staphylococcus phage mixture, both forming plaques on solid media in the seventh decimal dilution ( $10^{-7}$ ). *Staphylococcus aureus* "strain F" was used for their propagation in the course of the experiments. Polyvalent *B. coli* phage "MK" of MacNeal formed plaques on solid media in the sixth decimal dilution ( $10^{-6}$ ). Colon bacillus "strain I" was used for its propagation. (A) polyvalent pyocyaneus stock mixture phage of MacNeal which formed plaques on solid media in the sixth decimal dilution ( $10^{-6}$ ) was used with *B. pyocyaneus* "strain D."

## TECHNIQUE

In order to test as nearly as possible the actual concentration of the antiseptic commonly used for treatments, we employed 1 c.c. of ten times concentrated savita broth, to which was added 9 c.c. of antiseptic of the desired strength. Prontylin and sulfapyridine were dissolved directly in 1 per cent savita broth and zinc peroxide (Du Pont medicinal grade Z P O) was suspended in savita broth as well as in distilled water. The effect of the surgical solutions was studied both with and without savita broth. One tenth cubic centimeter of staphylococcus phage or 0.25 c.c. of *B. coli* or *B. pyocyaneus* phages was added to 10 c.c. of media and enough bacteria to make a final suspension of 50 million per c.c. throughout the experiments (see Figs. 1 and 2).

Our experiments differed from those of previous workers in that we demonstrated the complete inhibition or the diminution of the titer of the phages both in liquid and on solid media. The effect of sulfanilamide, sulfapyridine, and zinc peroxide is shown for the first time.

## RESULTS

The results of our experiments are presented in four groups. The first group embraces 11 antiseptics which destroyed staphylococcus, *B. coli*, and *B. pyocyaneus* bacteriophages and bacteria equally well within twenty four hours. This group includes 95 per cent and 10 per cent carbolic acid, 10 per cent argyrol (solargentum), 70 per cent alcohol, 2 per cent malachochrome, 35 per cent iodine, 1:500 and 1:5,000 dilutions of metaphen in water, 1 per cent acetic acid, 5 per cent carbolic acid and 5 per cent aluminum acetate.

TABLE I

TITER AND LYTIC ABILITY OF STAPHYLOCOCCUS, *B. COLI* AND *B. PYOCYANEUS* PHAGES AFTER 24 HOUR CONTACT WITH THE ANTISEPTICS WHICH DIFFERENTIALLY OR PARTIALLY INACTIVATED THE PHAGES

	STAPH. PHAGE		<i>B. COLI</i> PHAGE		<i>B. PYOCYANEUS</i> PHAGE	
	TITER	LYTIC ABILITY	TITER	LYTIC ABILITY	TITER	LYTIC ABILITY
Dakin's solution	0	0	0	0	4	4+
1:1,000 Merthiolate	0	0	0	0	4	4+
2% Gentian violet	0	0	u	0	u	0
1:500 Azochloramide	0	0	2	4+	1	4+
10% Zinc peroxide	0	0	3	-	1	-
1:2,000 Methylene blue	0	0	2	0	4	4+
25% Alcohol	0	0	1	3+	3	4+
Red wash 1 per cent zinc sulfate	0 or 1	0	3	0	4	±
1:10,000 Silver nitrate	0 or 1	0	3	4+	4	4+
1:1000 Hexylresorcinol	0	0	4	4+	4	4+
1% Zinc peroxide	0	0	4	-	4	-
1% Novocain	0 or 2	0	4	4+	4	4+
1:10,000 Merthiolate	0	0	5	4+	-	-
0.5% Carbolic acid	1	-	5	-	-	-
4% Boric acid	2 or 3	4+	1 or 4	4+	3 or 4	4+
2% Boric acid	3 or 4	4+	4	4+	4	4+
Savita control	7	4+	6	4+	6	4+

0 = Inability to recover bacteriophage (titer)

u = Recovery of phage from undiluted filtrate

1 up to 7 =  $10^1$  up to  $10^7$  phage titer on solid medium

4+ = Complete visible lysis in broth

3+ = Partial lysis

0 = No lysis

- = Not tested.

The second group included those found to have a differential or partial, lethal effect on our phages, as shown on Table I. This includes, in the order of their deleterious effect: Dakin's solution, 1:1,000 merthiolate, 2 per cent gentian violet, 1:500 azochloramide, 10 per cent zinc peroxide, 1:2,000 methylene blue, 25 per cent alcohol, red wash (1 per cent zinc sulfate), 1:10,000 silver nitrate, 1:1,000 hexylresorcinol, 1 per cent zinc peroxide, 1 per cent novocain, 1:10,000 merthiolate, and 4 per cent boric acid.

Most of the antiseptics of this group produced complete destruction of staphylococcus bacteriophage and only partial destruction of *B. coli* and *B. pyocyaneus* phages. *B. pyocyaneus* phage in general proved to be more resistant to the effect of antiseptics than *B. coli* phage.

Of particular interest to us was the effect of 1 per cent novocain which was found to possess a highly destructive action against staphylococcus phage diminishing the titer from  $10^7$  to  $10^2$ . There was also a decrease of titer from  $10^7$  to  $10^4$  of both *B. coli* and *B. pyocyaneus* phages.

Five-tenths per cent carbolic acid and 2 per cent and 4 per cent boric acid represent the weakest among the commonly used surgical antiseptics. They produced only a partial destruction of staphylococcus, *B. coli*, and *B. pyocyaneus* phages.

TABLE II

TITER AND LYTIC ABILITY OF STAPHYLOCOCCUS, *B. COLI*, AND *B. PYOCYANEUS* PHAGES AFTER 24-HOUR CONTACT WITH PRONTYLIN, PRONTOSIL, AND SULFAPYRIDINE

	STAPH PHAGE		<i>B. COLI</i> PHAGE		<i>B. PYOCYANEUS</i> PHAGE	
	TITER	LYTIC ABILITY	TITER	LYTIC ABILITY	TITER	LYTIC ABILITY
1:100 and 1:1,000 Prontylin	7 or 6	4+	6	4+	5 or 6	4+
1:10,000 and 1:1,000,000 Prontylin	7	4+	6	4+	6	4+
2.5% Prontosil	4	0	6	4+	6	4+
1%, 0.5% and 0.1% 1:10,000 and 1:33,000 Sulfapyridine	7 or 6	4+	6	4+	6	4+
Prontosil						
Savita control	7	4+	6	4+	6	4+

4, 6, 7 =  $10^4$ ,  $10^6$  and  $10^7$  phage titer on solid medium.

4+ = Complete visible lysis in broth.

0 = No lysis.

Table II presents the third group which shows results of the effect on phages of various dilutions of prontylin (sulfanilamide) and prontosil. In order to approach as nearly as possible the actual concentration of sulfanilamide in the body, a wide range of dilutions from 1:100 to 1:1,000,000 were used for the experiment. None of these dilutions was found to exert any deleterious effect on the titer or the lytic ability of *B. coli* phages, as compared with savita control. This was found to be the case after twenty-four hours of incubation, and again after one week and one month of refrigeration. A 1 per cent dilution of sulfanilamide was found to produce an occasional diminution of the titer of staphylococcus phage from  $10^7$  to  $10^6$  and of *B. pyocyaneus* phage from  $10^7$  to  $10^5$ . There was complete visible lysis by the treated phages in liquid medium. A 1 per cent dilution of sulfanilamide was also found to possess ant-

a slight bacteriostatic effect on all three types of bacteria and this was noticeable only during the first few hours of incubation. One tenth per cent and higher dilutions did not exert any visible effect either on phages or bacteria. Results with sulfanilamide in savita broth which was heated up to 50° C were very similar.

The effect of sulfapyridine was studied in dilutions 1:1,000, 1:10,000, and 1:33,000. These dilutions represent the range of concentration which sulfapyridine usually reaches in the blood of patients treated with this agent. None of these dilutions of sulfapyridine was found to exert any deleterious effect upon the lytic ability of staphylococcus *B. coli* and *B. pyocyaneus* bacteriophages. On the contrary, there was an indication that sulfapyridine enhanced the lytic effect of staphylococcus and *B. coli* phages. This study is to be presented in a separate publication. However, the titer of the bacteriophages propagated in the presence of sulfapyridine usually remained unchanged or fell slightly.

There was a definite bacteriostatic effect against *B. coli* and *B. pyocyaneus*, which was most pronounced within the first six hours. However, after twenty-four hours, the difference in degree of bacterial growth in tubes containing sulfapyridine and the controls was only slight or completely absent. No bacteriostatic effect was observed toward cultures of *Staphylococcus aureus*.

Compared with other dyes previously noted the antiseptic power of 2.5 per cent prontosil showed the least deleterious effect. Twenty-four hour contact of 0.1 cc of staphylococcus phage with 10 cc of 2.5 per cent prontosil resulted in lowering the titer of the phage from  $10^7$  to  $10^4$  and the lytic ability of the treated phage was completely inhibited. However, when 1 cc of phage was taken instead of 0.1 cc, the titer of the phage was reduced only from  $10^7$  to  $10^6$ , in other words, was practically unaltered. Twenty-four hour contact of 2.5 per cent prontosil with *B. coli* and *B. pyocyaneus* phages brought no change of titer or lytic ability to these phages. There was no visible effect on the development of staphylococcus, *B. coli*, or *B. pyocyaneus* organisms. One per cent, 0.5 per cent, and 0.1 per cent dilutions of prontosil were likewise found to produce no visible changes in staphylococcus, *B. coli*, and *B. pyocyaneus* phages or their homologous bacteria. In fact, titration of staphylococcus phage after twenty-four hour contact with 1 per cent and 0.5 per cent prontosil showed the titer to be slightly higher than that of the savita control, and the lysis in liquid medium took place a little before the savita control. The possibility of an adjuvant action of these dilutions on bacteriophage is being studied and will be reported later.

The surgical solutions are included in the fourth group. Bacteriophages prepared in savita broth as well as in distilled water containing 1 per cent saline (sodium chloride) instead of 0.5 per cent, with one exception, differed in no respect from the control phages. The same was true for Ringer's solution, 5 per cent dextrose in distilled water, and 5 per cent dextrose in Ringer's solution.

There was only partial lysis of the staphylococcus in 5 per cent saline (sodium chloride) in savita broth, and the titer of the phage was found to be  $10^6$ . On the other hand, there was complete lysis with *B. coli* and *B. pyocyaneus* phages, although the titer of the phages was diminished in both cases to  $10^4$ .

## DISCUSSION

In this study we have attempted to find out what effect certain antiseptics and other dressing solutions, commonly used in surgical practice, had upon the potent staphylococcus, *B. coli*, and *B. pyocyaneus* bacteriophages. We have included a number of surgical solutions employed locally or parenterally, and novocain, which is sometimes used as a preliminary medication.

Comparing the results of previous workers with our own, we find a number of apparent discrepancies. Some of these seem to be due to differences in technique, such as variations in the medium in which phage is brought into contact with the antiseptic, its chemical reaction and its amount, as well as the time and temperature at which the experiments are carried out. But the chief discrepancy is probably due to the different types and the potency of the phages employed.

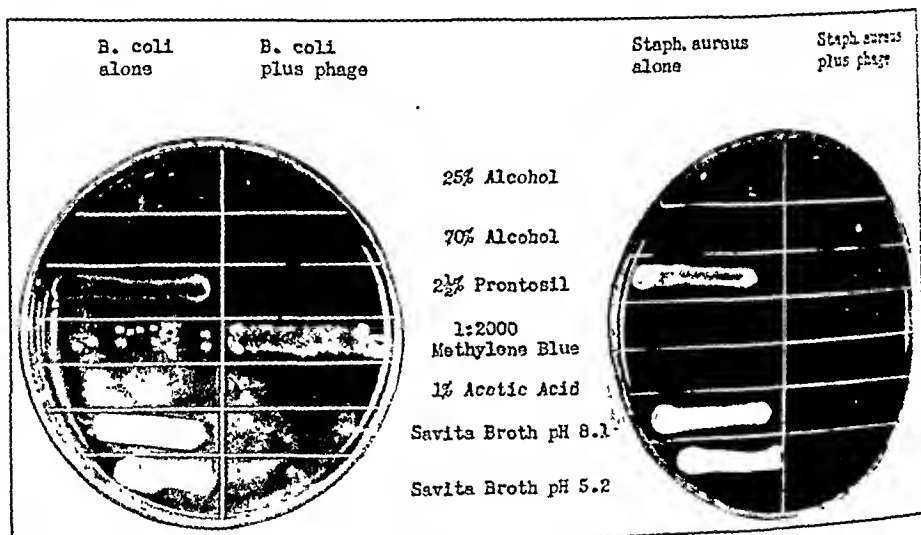


Fig. 1.—The comparative effect of various antiseptics on staphylococcus and *B. coli* phages and their corresponding bacteria after contact with 25 per cent and 70 per cent alcohol, 2.5 per cent protosil, 1:2,000 methylene blue, 1 per cent acetic acid, and 1 per cent broth of pH 8.1 and 5.2. It is seen that 25 per cent and 70 per cent alcohol, and 1 per cent acetic acid destroyed staphylococcus and *B. coli* organisms. Methylene blue almost completely inhibited the growth of staphylococcus, but a few colonies of *B. coli* survived. Two and a half per cent protosil did not prevent the normal growth of either bacteria, but interfered with the lytic action of staphylococcus bacteriophage, as evidenced by the partial growth on the plate. There was no apparent effect on *B. coli* phage. A few colonies of growth from staphylococcus phage control of pH 5.2 indicate that it is affected by the low pH of the medium as contrasted with no growth from the pH 8.1 control tube.

Our work has been in general agreement with the previous reports to the effect that bacteriophage is susceptible to, and is destroyed by, certain of the stronger antiseptics, and that there is a differential susceptibility to some of the weaker antiseptics, the staphylococcus phages being regularly more susceptible than the *B. coli* phages. We have also shown that the latter in turn are more susceptible than the *B. pyocyaneus* phages.

None of our experiments has thrown any light upon the reason for this differentiation. It is well known, of course, that different species of bacteria vary in their susceptibility to antiseptics and to a less degree different strains within the same species vary. The analogy may be an indication that the less

terio-phages are biologically similar to bacteria and behave more like living organisms in this respect than chemical reagents or even enzymes. It is of some interest that in general the bacteria are more susceptible than their corresponding phages. Potency does not seem to run parallel with susceptibility, because the two staphylococcus phages differed in potency but were similar in susceptibility. Also, the staphylococcus phages were both more polyvalent and more potent than the *B. coli* phages although the latter were more resistant. These in turn were more potent than the more resistant *B. pyocyaneus* phage mixture.

We have observed that the staphylococcus phages do not propagate as rapidly at low pH levels as the other two phages and it may be that the low pH of most of the antiseptics may play a part in the easier destruction of the staphylococcus phages. However, staphylococcus phages were also more susceptible to the zinc peroxide suspensions which are on high pH levels. This agrees with Sierakowski and Zabłudowska<sup>36</sup> who believe that the high pH ranges are also inhibitors of staphylococcus phage. They showed that staphylococcus phage was killed at pH 9.45, while *B. coli* phage was destroyed at pH 10.3. The chief dis-

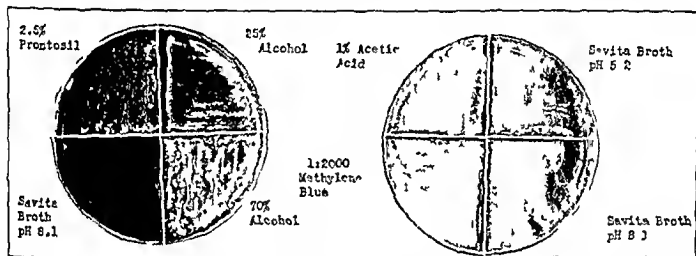


Fig. 2.—The potency of staphylococcus phage  $\alpha$  estimated by titration on agar plates. Normal growth of staphylococcus on quadrants inoculated with bacteria and with a loopful of undiluted phage filtrate after contact with 25 per cent and 0 per cent alcohol, 1 per cent acetic acid and 1:2000 methylene blue show that bacteriophage is completely inactivated. Numerous plaques of phage replacing bacterial growth as the result of phage action after contact with 25 per cent protosil show that bacteriophage is present but contains a smaller number of corpuscles than savita phage control. Phage grown in savita broth of pH 8.1 obviously contains a larger number of corpuscles causing greater destruction of bacteria than savita control of pH 5.2. The dark area on the methylene blue quadrant is the result of the coloration of the agar.

crepancies in our results as compared with those of other workers have been in connection with the dyes methylene blue, metaphen, and merthiolate, where there has been a difference of opinion expressed by a number of authors. These discrepancies may be explained by variations in the susceptibility of different races of bacteriophage or by differences in the technique employed in the tests. This is of some practical importance because commercial phages which we studied had been "preserved" with merthiolate, and we were unable to propagate them, indicating that they had not been preserved but killed by the antiseptic.

Our results seem to indicate that certain of the weaker antiseptics may have a dual action on bacteriophage: first, an inhibition of propagation and second, an interference with its lytic action. By our tests, which have been checked on both liquid and solid media, we have frequently shown that the titer has been considerably lowered by the antiseptic, in other words, that propagation has

been delayed or stopped altogether while the surviving amounts of phage are fully capable of producing complete lysis. The explanation for this is not clear.

We have felt that in these experiments the value of determining the presence of phage on solid media was more important than the lytic test in liquid media. Frequently we have demonstrated the presence of plaques on plates when the corresponding dilutions of phage failed to clear the media. This is probably due to the fact that the optimum relationship between the number of bacteria and phage corpuscles did not exist, because potent phage could always be recovered from the plaques developing on the plates. With certain phages the clearing mechanism in liquid media cannot function unless there is a fairly high number of bacteriophage corpuscles present.

The most important part of our study, and one which has not been reported by other authors, has been the observations of the effect of prontosil, sulfapyridine, and prontosil. Our finding that neither of these potent "streptococides" interferes with phage action makes it possible to combine the use of sulfanilamide and sulfapyridine with phage in those mixed infections which are caused by the hemolytic streptococcus combined with staphylococcus or *B. coli*, and we have used this combined method with gratifying results in a number of instances.

One of our experiments suggested that diluted prontosil might even have an adjuvant action on bacteriophage as the titer was higher after contact with 0.5 per cent and 1.0 per cent prontosil. Sulfapyridine, on the other hand, although it occasionally decreased the bacteriophage titer, definitely increased the lytic activity of bacteriophage. This action will be studied further.

The only other antiseptics which will permit this procedure are 2 per cent and 4 per cent boric acid, which are of questionable antiseptic value. Figs. 1 and 2 also indicate clearly that prontosil has little or no direct antiseptic action on the organisms which we have tested.

One per cent prontosil (which is considerably more concentrated than it ever is when it reaches the lesion in the human body) seemed to produce some inhibition of growth of staphylococcus for the first four hours in liquid media, but did not prevent ultimate growth.

The complete inhibition of lysis by staphylococcus phage after contact with 2.5 per cent prontosil (which had no such effect on the *B. coli* and *B. pyocyaneus* phages) is another example of differential action which we have not been able to explain.

#### CONCLUSIONS

1. Antiseptics in general cannot be used with bacteriophage in the treatment of mixed infections. Zinc peroxide is no exception to this rule.
2. Sulfanilamide, sulfapyridine, and prontosil may be used with bacteriophage in the treatment of mixed infections.

#### REFERENCES

1. Kabeshima, T.: Sur le ferment d'immunité bacteriolysant. Compt. rend. Soc. de Biol. 83: 471, 1920.
2. Bablet, J.: Sur le principe bacteriophage de D'Herelle, Compt. rend. Soc. de Biol. 1322, 1920.
3. de Poorter, P., and Maisin, J.: Contribution à l'étude de la nature du principe bacteriophage, Arch. internat. de pharmacodyn. et de thérap. 26: 473, 1919-21.

- 4 d'Herelle, F Le bacteriophage son role dans l'immunité, Paris, 1921, Masson et Cie, p 277
- 5 Wolff, L, and Janzen, J Action de divers antiseptiques sur le bacteriophage d'Herelle, *Compt rend Soc de biol* 87 1087 1922
- 6 Tomaselli, C Contributio allo studio del batteriophage, Milano, 1923, A Cordiani, p 53
- 7 d'Herelle, F Action du fluorure de sodium sur le bacteriophage, *Compt rend Soc de biol* 88 407, 1923
- 8 Brutsaert P Les bacteriophages dans les milieux fluores, *Compt rend Soc de biol* 89 1173, 1923
- 9 Watanabe, T The Effect of Disinfectants on Bacteriophage, *Arch f Hyg* 92 1, 1923 24
- 10 Korb, C and Bronfenbreunner, J Effect of Alcohol on the So called Bacteriophage of d'Herelle, *Proc Soc Exper Biol & Med* 21 177, 1924
- 11 Stassano, H, and de Beaufort, A G Action du citrate de sand le principe lytique transmissible, *Compt rend Soc de biol* 93 1340 1924
- 12 Stassano, H, and de Beaufort A G L action de l'ether sur le principe lytique transmissible, *Compt rend Soc de biol* 93 1387 1926
- 13 Alessandrini A Action of Sodium Chloride on Bacteriophage Policlinico Rome 32 1705, 1925, *Abst J A M A* 86 518, 1926
- 14 d'Herelle, F The Bacteriophage and Its Behavior Baltimore 1926 Williams & Wilkins Co, pp 67 69
- 15 Brutsaert, P Les bacteriophages et les miers les dans le bouillon hypersale, *Compt rend Soc de biol* 90 646 1924
- 16 Schultz, E W, and Krueger, A P Inactivation of Staphylococcus Bacteriophage by Methylene Blue *Proc Soc Exper Biol & M* 1 27 1941 1928
- 17 Clifton, C E, and Lawler, T G Inactivation of Staphylococcus Bacteriophage by Toluidine Blue, *Proc Soc Exper Biol & Med* 27 1941 1930
- 18 Clifton, C E Photodynamic Action of Certain Dyes on the Activation of Staphylococcus Bacteriophage, *Proc Soc Exper Biol & Med* 28 745 1931
- 19 Wells, A T, and Sherwood, N P Selective Action of Crystal Violet and of Brilliant Green on Bacteriophages, *J Infect Dis* 52 209 1933
- 20 Wells, A T, and Sherwood N P Selective Action of Dyes and of Other Disinfectants on Bacteriophages, *J Infect Dis* 55 195 1934
- 21 Walker, A W Comparison of Precipitating Action of Basic Dyes on Bacteriophage and Bacterial Proteolytic Enzymes *Proc Soc Exper Biol & Med* 34 726, 1936
- 22 Arlong, F, and Langeron, L Action de l'eau le javel sur diverses especes de bacteriophages, *Compt rend Soc de biol* 96 434 1927
- 23 Frisbee, F C, and McNeal, W J The Behavior of *Escherichia coli* and Its Specific Bacteriophage in Urine The Influence of Environmental Factors, *J Infect Dis* 46 404, 1930
- 24 Jamieson, W A, and Powell H M Merthiolate as a Preservative for Biological Products, *Am J Hyg* 14 218, 1931
- 25 Powell, H M, Jamieson, W A, and James, F G Merthiolate as Preservative for Biological Products III Action of Merthiolate on Bacteriophage, *J Immunol* 24 185, 1933
- 26 Clifton, C E Preservatives for Bacteriophage Suspensions, *Proc Soc Exper Biol & Med* 29 370, 1931 32
- 27 Rikieten, M L The Preservation of Polyvalent Staphylococcus Bacteriophage, *Science* 76 85, 1932
- 28 Straub, M E, and Applebaum, M Studies on Commercial Bacteriophage Products, *J A M A* 100 110, 1933
- 29 Krueger, A P, and Elberg, S Reversible Inactivation of Bacteriophage by KCN, *Proc Soc Exper Biol & Med* 31 483, 1934
- 30 Krueger, A P, and Baldwin D M The Reversible Inactivation of Bacteriophage With Sulfurine, *J Infect Dis* 57 207, 1935
- 31 Goldsmith, N R Differences in Effect of Phenylmercuric Chloride Upon Different Races of Bacteriophage and Similarity in Effect Upon a Phage and Its Homologous Organisms, *J Bact* 30 237, 1935
- 32 Lominski, I Inactivation of Bacteriophage by Oxidation, *Compt rend Soc de biol* 122 766, 1936
- 33 Lominski, I Inactivation of Bacteriophage by Oxidation Reaction by Ascorbic Acid, *Compt rend Soc de biol* 119 1345, 1935
- 34 Lominski, I Inactivation of Bacteriophage by Ascorbic Acid, *Compt rend Soc de biol* 122 766, 1936
- 35 Guller, W Behavior of Different Bacteriophages Against Chemical and Physical Agents, *Ztschr f Immunitatsforsch u exper Therap* 86 248, 1936
- 36 Sierakowski, S, and Zabłudowska, F Le pH limite des bacteriophages, *Compt rend Soc de biol* 103 422, 1930



37. Jern, H. Z., Howes, E. L., and Meleney, F. L.: Studies in Bacteriophage. I. The Behavior of the Bacteriophage and the Bacteria in the Lesion After the Treatment of Acute Staphylococcus Skin Infections With Bacteriophage, *J. LAB. & CLIN. MED.* 19: 1257, 1934.
38. Jern, H. Z., and Meleney, F. L.: Studies in Bacteriophage. II. Methods of Marking and Testing the Potency of Bacteriophage, *J. LAB. & CLIN. MED.* 22: 1935.
39. Jern, H. Z., and Meleney, F. L.: Studies in Bacteriophage. III. The Significance of Tests for the Inhibition of the Bacteriophage Phenomenon by Human Serum, *J. LAB. & CLIN. MED.* 22: 284, 1936.
40. Domagk, G.: Ein Beitrag zur Chemotherapie der bakteriellen Infektionen, *Deutsch. med. Wchnschr.* 61: 250, 1935.
41. Colebrook, L., Buttle, G. H. A., and O'Meara, R. A. Q.: Mode of Action of P-aminobenzenesulphonamide and Prontosil in Hemolytic Streptococcus Infections, *Lancet* 2: 1323, 1936.
42. Long, P. H., and Bliss, E. A.: P-aminobenzenesulphonamide and Its Derivatives. Experimental and Clinical Observations on Their Use in Treatment of Hemolytic Streptococcal Infections, *J. A. M. A.* 100: 32, 1937.
43. Meleney, F. L.: Zinc Peroxide in the Treatment of Micro-aerophilic and Anaerobic Infections With Special Reference to a Group of Chronic Ulcerative Burrowing and Gangrenous Lesions of the Abdominal Wall Apparently Due to a Micro-aerophilic Hemolytic Streptococcus, *Ann. Surg.* 101: 997, 1935.
44. Meleney, F. L., and Johnson, B. A.: The Prophylactic and Active Treatment of Surgical Infections With Zinc Peroxide, *Surg., Gynec. & Obst.* 64: 357, 1937.
45. Meleney, F. L., and Johnson, B. A.: Further Laboratory and Clinical Experience in the Treatment of Chronic, Undermining, Burrowing Ulcers With Zinc Peroxide, *Surgery* 1: 169, 1937.

## THE RATIONALE OF THE USE OF TESTOSTERONE PROPIONATE IN FUNCTIONAL UTERINE BLEEDING AND DYSMENORRHEA\*

H. S. RUBINSTEIN, M.D., BALTIMORE, MD.

IT HAS recently been observed that testosterone propionate when administered in adequate dosage to patients with functional uterine bleeding or functional dysmenorrhea<sup>1-4</sup> leads to cessation of the bleeding and to an alleviation of the pain. It is difficult in the present state of knowledge to explain exactly why the male sex hormone favorably influences such bleeding and pain. Chemically testosterone propionate is quite similar in structure to progesterone<sup>5</sup> (Fig. 1). It is known that normally progesterone becomes an important factor after ovulation when further follicle effect is held in abeyance. One is, therefore, led to assume that suppression of overactivity of the Graafian follicle is an important factor in maintaining a normal menstrual flow. This concept suggests itself particularly when one realizes that persistent follicle cysts are frequent accompaniments of menorrhagic states and that functional dysmenorrhea has frequently been associated with excessive estrogen concentration in blood and urine.<sup>6, 7</sup> Progesterone has been found to retard follicle maturation and to inhibit ovulation.<sup>8</sup>

Experiments conducted in this laboratory suggest that testosterone propionate has a similar effect. When testosterone propionate (Perandren, Ciba) was given to immature female rats 21 days of age in 1 mg. daily intraperitoneally admini-

\*From the Laboratory for Research, Surgical Division, Sinai Hospital.  
Received for publication, November 8, 1938.

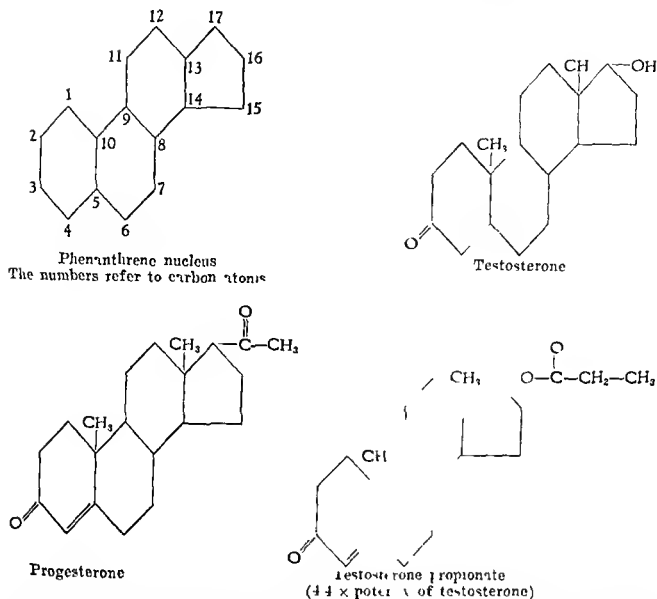


Fig 1—Showing the close chemical relationship between testosterone propionate and progesterone. Both contain oxygen on carbon atom No 3, a double bond between atoms 4 and 5 and  $\text{CH}_3$  groups at atoms 10 and 13. Thus the basic ring structure is similar. The difference is found in the grouping on the No 17 carbon.

tered doses, it was found that notwithstanding the fact that premature vaginal opening resulted at 30 days of age in test animals as compared to 38 days for the controls, the vaginal smears at the time of opening showed only a proestrus picture<sup>o</sup>. When sacrificed forty eight hours later (32 days of age), their ovaries showed no evidence of ovulation. As a matter of fact, the ovaries of the test animals were smaller and the follicles were less mature than those found in the control (Fig 2). In another group of animals in which similar injections were begun at 21 days of age and continued to the age of 67 days, it was found that the vaginas of this treated group also opened at 30 days of age, again displaying a proestrus smear, while vaginal opening in the controls occurred on the thirty eighth day, at which time these animals also showed proestrus smears. Daily vaginal smears taken in all animals from the time of vaginal opening to the sixty seventh day disclosed a normal five to six day complete estrus cycle in the controls while the test animals showed either diestrus or proestrus pictures. At autopsy the ovaries of the controls were again found to be larger than those of the test animals and were seen to contain large corpora lutea. No evidence of ovulation or luteinization was found in the ovaries of the injected animals (Fig 1). This suggests a suppression of ovulation in the test animals as a result of repeated injections of testosterone propionate.



Fig 2—Comparison of ovaries of animals (rats) treated with testosterone propionate and their controls (magnification  $\times 70$ ). A, Ovary of 32-day-old treated rat, obtained forty eight hours after artificially induced vaginal opening. The ovary is smaller and the follicles are at a lower stage of development than that seen in the control. No corpora lutea are visible. B, Ovary of 32-day-old control rat obtained while vagina was still unopened. The ovary is relatively large and the follicles appear highly developed. No corpora lutea are visible. C, Ovary of 67-day-old treated rat. Ovary is relatively small, no definite corpora lutea can be distinguished. D, Ovary of 67-day-old control. The specimen is twice the size of that seen in the treated animal. Several huge corpora lutea are present, follicle maturation is progressing normally.

Hence, it is possible that the similarity in chemical structure between testosterone propionate and progesterone may explain the progesterone-like effect of the substance used.

It will be noted from previous reports<sup>3, 4</sup> that the dosage which was successful in our cases was much smaller than that recently reported by others.<sup>1, 2</sup>

With doses of 500 mg or more (using 15 mg intramuscularly every other day), Loesser (1938) noted a suppression of menstruation which persisted for six to ten weeks after cessation of injections. Foss (1938) also observed amenor-

rhea following the use of 100 mg daily. It appears that in spite of the fact that these authors noted no untoward effects following their large dosage, smaller amounts are more physiologic since menses are more normally established and maintained.

The cause of amenorrhea following injections of large doses of testosterone is probably inhibition of the pituitary gland with a suppression of output of pituitary gonadotropic substance.<sup>10</sup>

#### CONCLUSION

The rationale of the use of testosterone propionate in the treatment of functional uterine bleeding and functional dysmenorrhea is considered. It is felt that, due to its close chemical relationship to progesterone, testosterone propionate inhibits follicular maturation and ovulation and locally leads to relaxation of the myometrium. In addition in very large doses it inhibits the gonadotropic secretions of the anterior pituitary gland.

Estrogenic concentration is, therefore, diminished. This favors uterine relaxation and checks the continuation of the interval endometrium, thus altering the two common immediate causes of excessive pain and bleeding.

#### REFERENCES

- 1 Loesser, A. A. The Action of Testosterone Propionate on the Uterus and Breast, *Lancet* 234: 375, 1938.
- 2 Foss, G. L. The Action of Testosterone Propionate on the Female, *Lancet* 234: 992, 1938.
- 3 Rubinstein, H. S., and Abarbanel, A. R. The Use of Testosterone Propionate in the Treatment of Dysmenorrhea, *Am J Obst & Gyn* 37: 701, 1939.
- 4 Rubinstein, H. S. Testosterone Propionate in the Treatment of Functional Uterine Bleeding, *J A M A* 112: 537, 1937.
- 5 Fieser, L. F. *The Chemistry of Natural Products Related to Phenanthrene*. New York, 1937, Reinhold Publishing Corporation.
- 6 Kotz, J., and Parker, E. Primary Dysmenorrhea—An Endocrine Problem, *Am J Obst & Gynec* 34: 38, 1937.
- 7 Smith, G. V., and Smith, O. W. Urinary Excretion of Estrogenic and Gonadotropic Hormones During Normal Menstrual Cycle, Periods of Conception and Early Pregnancy, *New England J Med* 215: 908, 1936.
- 8 Makepeace, A. W., Weinstein, J. L., and Friedman, M. H. The Effect of Progesterone on Ovulation in the Rabbit, *Am J Physiol* 119: 512, 1937.
- 9 Rubinstein, H. S., Abarbanel, A. R., and Nader, D. H. The Influence of Testosterone Propionate on Vaginal Opening in the Immature Albino Rat, *Proc Soc Exper Biol & Med* 39: 20, 1938.
- 10 Hamilton, J. B., and Wolfe, J. M. The Effect of Synthetic Androgen Upon the Gonadotropic Potency of the Anterior Pituitary, *Endocrinology* 22: 360, 1938.

The author gratefully acknowledges the cooperation of Ciba Pharmaceutical Products, Inc. for partially defraying the expenses involved in this study.

## EXPERIENCE WITH ZINC INSULIN CRYSTALS\*

CHARLES M. LEVIN, M.D., ELMER A. KLEEFIELD, M.D., AND  
FRANK A. LUCIANO, M.D., JAMAICA, N. Y.

THE advent of protamine zinc insulin was a distinct advance in the treatment of diabetes mellitus. The diabetic who required insulin became more fortunate. His insulin administration was reduced to one injection per day, and the total amount of insulin required was often less than that of standard insulin. In many instances the food intake, particularly carbohydrate, could be increased above the previous allowance, and a certain amount of variation in the feeding time schedule was permissible. On the whole, the outlook for the diabetic was a happier one.

However, with the continued use of protamine zinc insulin in medium and severe diabetic patients, disturbing problems arose in the control of many of these individuals. Situations similar to those met by Ralli<sup>1</sup> and others were encountered. Commonly, of course, protamine zinc insulin when given alone did not always control early and forenoon hyperglycemia and glycosuria. On the other hand, the failure of a given supplement of standard insulin to control adequately and consistently the blood sugar fluctuations during this period was not rare. Some patients varied in their regulation from day to day to such an extent that some authorities wondered whether or not hyperglycemia and glycosuria were of any importance under these circumstances. We are in accord with Kepler<sup>2</sup> who states: "Nevertheless simply because we cannot always achieve a sugar free urine with the use of protamine zinc insulin, we as physicians are not justified in abandoning our former position that consistently sugar free urine is the 'sine qua non' in the treatment of diabetes." Wilder<sup>3</sup> also has said: "The degree of glycosuria under these circumstances may be harmless but until we have proof of its harmlessness we must insist on its control."

In the second place qualitative and quantitative rearrangements in the diet in order to meet the challenge of varying blood sugar levels became hardships to many individuals whose eating habits and working hours made such changes uncomfortable or inconvenient. For many juvenile diabetics the late evening feedings recommended were sometimes difficult problems. Discussing various types of dietary formulas for diabetics on protamine zinc insulin, Wilder<sup>4</sup> says: "These procedures undoubtedly are useful in tricky cases. I object to them for routine management because of the inconvenience they cause the patient. So far as possible we should strive to interfere as little as necessary with customary habits of eating. . . ."

\*From the Metabolic and Nutrition Division, Department of Medicine, Queens General Hospital.

Received for publication, November 28, 1935.

The solution of zinc insulin crystals used in this study was generously supplied by Frederick Stearns & Co., of Detroit. This material was formerly known as crystalline insulin.

Lastly, in those diabetics whose urines *were* kept consistently sugar free the problem of night hypoglycemia often arose. Even an intelligent experienced diabetic has difficulty in combating protamine insulin reactions of notoriously insidious onset and prolonged hypoglycemic effect.

Since protamine zinc insulin did not solve all the problems encountered in the use of standard insulin, it was often felt that an insulin whose action was intermediate between these two might make our armamentarium for treating the diabetic more complete.

Crystalline insulin, first isolated by Abel of Johns Hopkins, was prepared for the first time in amounts large enough for clinical investigation by Melville Sayhoun of the Stearns Laboratories. In 1936 Freund and Adles<sup>5</sup> demonstrated that crystalline insulin had an onset of action more rapid than that of protamine insulin but was effective two or three times longer than standard insulin. The number of injections and units required were often less than for standard insulin. Mains and McMullen<sup>6</sup> and Altshuler and Leiser<sup>7</sup> came to similar conclusions. Wilder<sup>4</sup> also reported the action of crystalline insulin to be intermediate to the other two insulins. Barach<sup>8</sup> finding this insulin to be an improvement over standard insulin in 12 out of 21 cases, noted that reactions occurred only one fourth as frequently as with standard insulin.

Why the crystalline insulin has a prolonged action has not yet been definitely determined. Experiments have shown that the amount of zinc in the crystalline insulin solution is not a factor.<sup>9</sup> Sayhoun<sup>10</sup> believes that the relative insolubility of the crystals at the tissue pH is responsible.

In February, 1938, we began to observe the action of crystalline insulin, now known as solution of zinc insulin crystals, in diabetic patients. For certain difficult cases, an insulin which would prevent extreme and rapid oscillations between hyperglycemia and hypoglycemia, or which would not permit prolonged hyperglycemia followed by prolonged hypoglycemia, might prove useful. Reduction in the number of doses of insulin below the customary number of standard insulin injections or reduction in the total daily amount of insulin needed were only secondary considerations. Primarily, we were interested in finding some thing which would keep the diabetic patient's blood sugar within physiologic ranges throughout the entire day.

Uncomplicated hospitalized diabetics ranging in age from 12 to 60 years, were chosen. Older patients were not selected because they usually have mild diabetes, take little insulin, and the question of fluctuations is not a prominent factor.

As a preliminary study, we compared the actions of standard insulin, protamine zinc insulin, and zinc insulin crystals respectively, in three apparently normal individuals. These subjects were each given diets of 250 gm carbohydrates, 80 gm protein, and 130 gm fat, divided into three equal meals. Each individual's twenty four hour blood sugar curve was first estimated on diet alone. We then gave single doses of 10 units of standard insulin, zinc insulin crystals, and protamine zinc insulin, respectively, for one day each. Four days were allowed to elapse between each series of blood sugar determinations in order to obviate any possible effect of the previous dose of insulin. In all three subjects it was evident as already established that the maximum effect of standard insulin

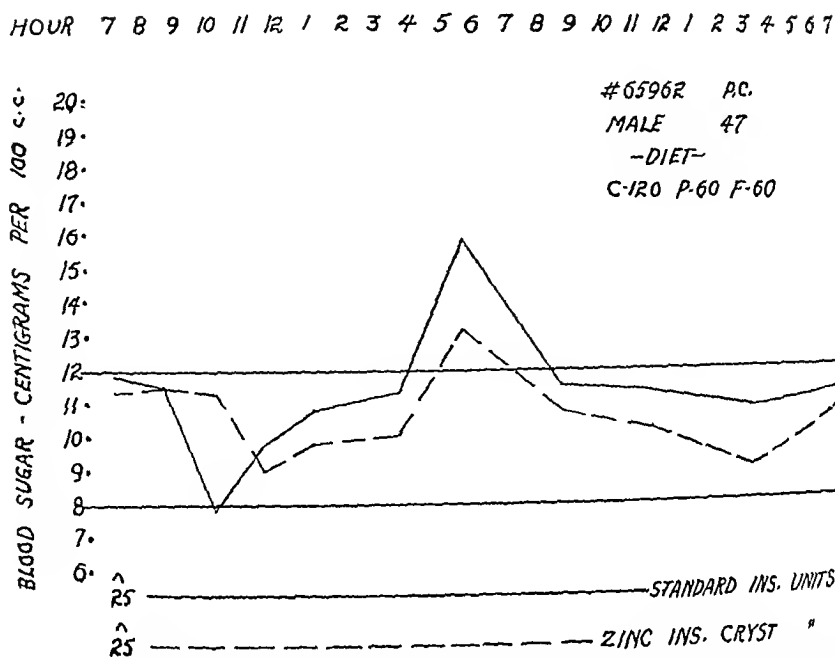


FIG. 1.—On one dose of standard insulin sharp effect of insulin at 10 A.M., with high peak at 5:30 P.M. On same dose of zinc insulin crystals, tendency to follow blood sugar curve as with standard insulin. However, only slight peak at 5:30 P.M., with generally more even stabilization.

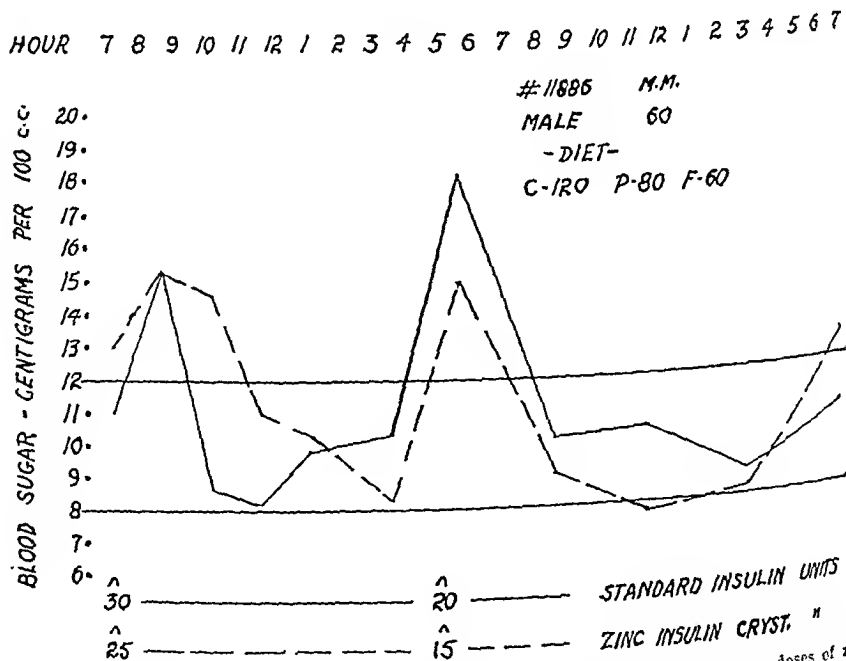


FIG. 2.—With two doses of standard insulin, fluctuations evident. On two doses of zinc insulin crystals with reduction of total dosage by ten units, an improved curve. A postprandial fall instead of rise at 1 P.M. with zinc insulin crystals. Note how crystalline curve otherwise follows standard curve.

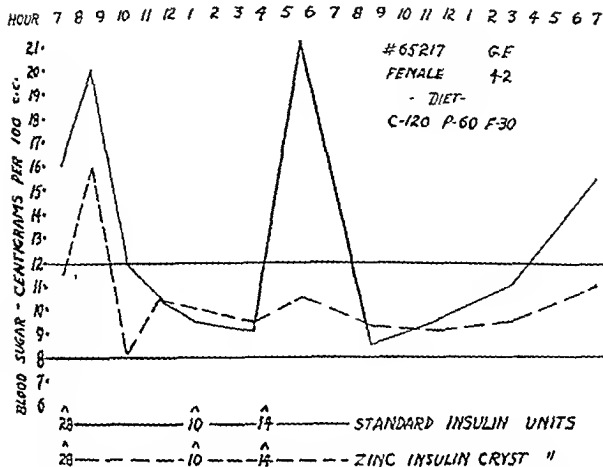


Fig. 3—On three doses of standard insulin, wide fluctuations. On equal doses of zinc insulin crystals marked leveling off with uniformly stable blood curve.

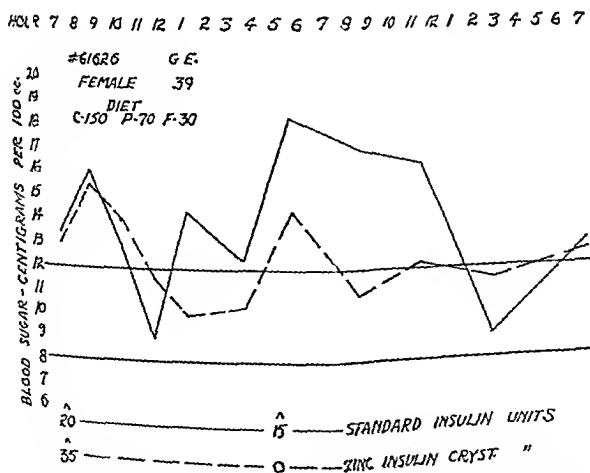


Fig. 4—On two doses of standard insulin markedly irregular curve, although sugar free and ordinarily considered good control. On one dose of zinc insulin crystals equal to the sum of the two doses of standard, a very marked improvement in blood curve and evident physiologic control.



HOUR 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1

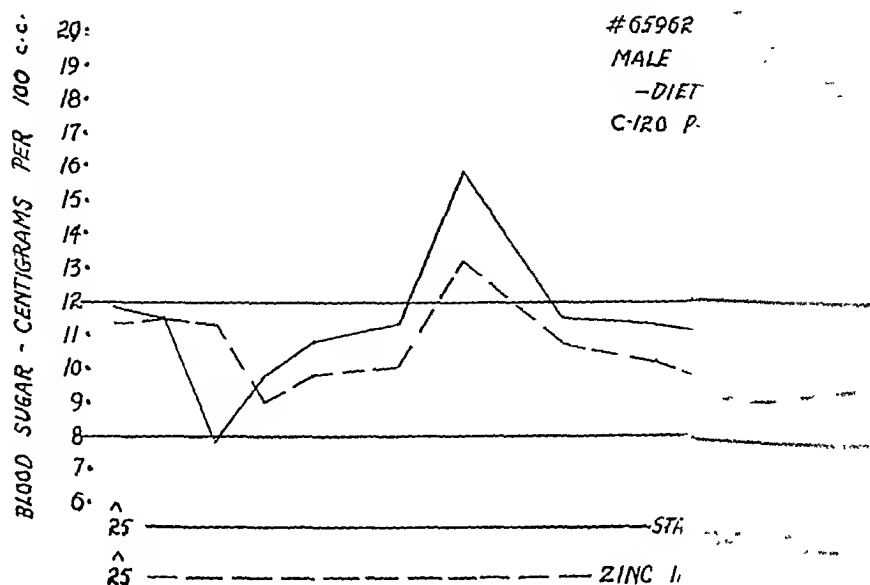


Fig. 1.—On one dose of standard insulin sharp effect of insulin at 5:30 P.M. On same dose of zinc insulin crystals, tendency to follow standard insulin. However, only slight peak at 5:30 P.M., with general

HOUR 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12

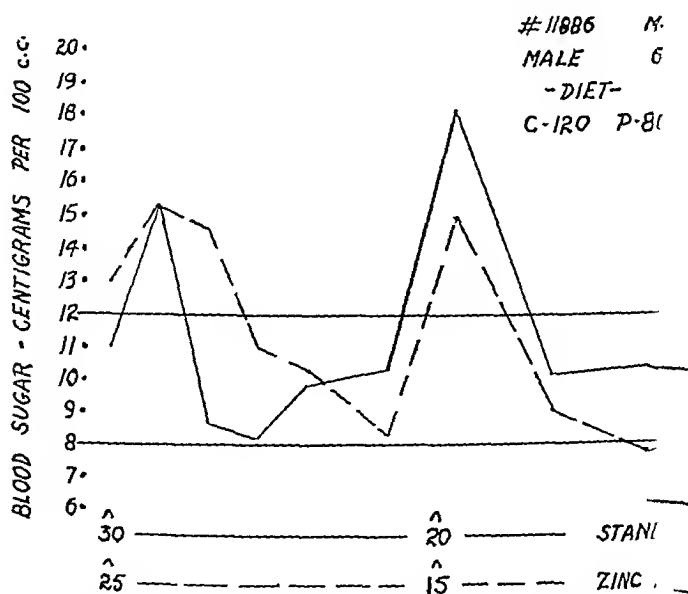


Fig. 2.—With two doses of standard insulin, fluctuations evident. With zinc insulin crystals with reduction of total dosage by ten units, a prandial fall instead of rise at 1 P.M., with zinc insulin crystals. Otherwise follows standard curve.

was four hours in length, that of protamine zinc insulin for twenty four or more hours, and that of zinc insulin crystals for fourteen hours.

In diabetic patients, we feel that as Suddon<sup>11</sup> has emphasized, the frequently sharp and high postprandial rises are important. A test of the zinc insulin crystals would be whether or not these particular oscillations were affected. In addition the interval between 7 at night and 7 in the morning should receive more consideration. Unless blood sugars are determined with reasonable frequency, this period may be deceptive. For these reasons the series of blood sugars determined on each patient included samples one and one half hours after meals and several determinations throughout the night. Blood for sugar determination by the modified Folin micromethod was taken at 7 30, 10, and 11 30 AM and 1, 3, 5 30, 8 30, and 11 30 PM and at 3 and 7 o'clock the succeeding morning. These samples were all taken by two of us and all colorimetric determinations were made by one of us.

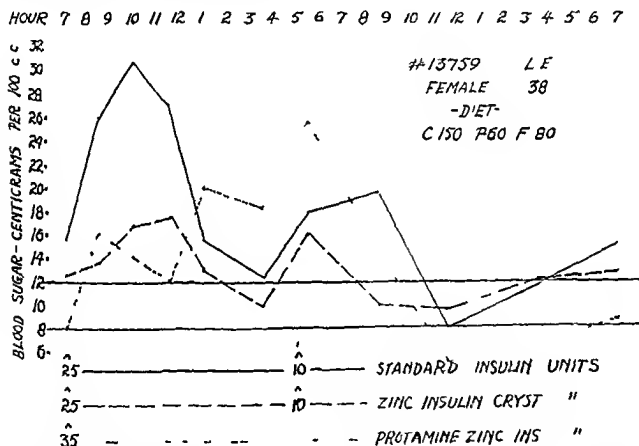


Fig. 7.—Regulation with standard insulin poor. One lot of protamine zinc insulin equivalent to the total of standard did not improve the blood sugar. On two lots of zinc insulin crystals equivalent to the standard insulin a physiologic blood curve resulted. The patient felt entirely normal on this regulation for the first time in his experience with insulin.

Because of hospital routine, the diets were served at 7 15 AM, 11 AM, and 4 PM having been divided into three equal meals unless protamine zinc insulin was being used. In such instances the carbohydrate was divided as follows: one sixth for breakfast, one third each for lunch and supper, and one sixth at about 8 30 PM.

In many patients blood sugar curves were repeated without changing the type or dose of insulin in order to rule out the question of time under treatment as a factor in improved curves. Patients showing variable blood sugar curves on fixed doses of insulin were excluded from this study. In all, a total of 1,866 blood sugar determinations were performed. Figs. 1 to 8 are examples of various methods used to establish best regulation.



a duration of action intermediate between that of standard insulin and protamine zinc insulin. This action was effective in most instances from eight to sixteen hours.

In nearly all patients the amplitude of blood sugar fluctuations observed with standard insulin regulation was decreased when crystalline insulin was substituted. This was most noticeable in those patients who presented very marked fluctuations.

In all patients substitution of an equal amount of zinc insulin crystals for standard insulin produced improvement in the blood sugar graphs. When the dose of zinc insulin crystals was reduced, some patients still revealed some lowering of the blood sugar curve. In no case did standard insulin produce a more satisfactory result than did zinc insulin crystals.

Most of the patients voluntarily stated that they felt much better on zinc insulin crystals than on standard insulin. We believe that this is due to the sharper fluctuations produced by standard insulin. Patients with blood sugar levels of 50 mg rarely showed untoward symptoms when zinc insulin crystals were used, whereas there was definite shock when the same level was produced by standard insulin.

Where it was necessary to supplement protamine zinc insulin substitution of zinc insulin crystals for the standard insulin supplement usually produced a definite improvement in the blood sugar wave assuring the patient of a more stable regulation.

#### CONCLUSION

Solution of zinc insulin crystals is a valuable adjunct to the treatment of diabetes mellitus. Its action is much more prolonged and steadier than that of standard insulin, but is shorter in duration than that of protamine zinc insulin. It conveniently assumes the role of an intermediate between the latter two. In regulation it does what standard insulin does but apparently produces a less fluctuant type of blood sugar curve. As yet we have not familiarized ourselves with its action in diabetes with acidosis and so cannot comment on the efficiency of zinc insulin crystals in such situations.

#### REFERENCES

1. Ralli, E. P., Fern, H. D., and Lovelock, F. J. Observations on the Continued Use of Protamine Zinc Insulin in Patients With Severe Diabetes Mellitus, *Am J M Sc* 196 28, 1938.
2. Kepler, E. J. Clinical Experience With Protamine Zinc Insulin, *J A M A* 110 92, 1938.
3. Wilder, R. M. Clinical Investigations of Insulins With Prolonged Activity (Trimble Lecture), *Ann Int Med* 11 13, 1937.
4. Wilder, R. M. and Wilbur, D. L. Diseases of Metabolism and Nutrition, Review of Certain Recent Contributions. *Arch Int Med* 61 297, 1938.
5. Freund, H. A., and Adler, S. Effects of Standard Protamine and Crystalline Insulin on Blood Sugar Levels, *J A M A* 107 573, 1936.
6. Mains, M. P., and McMullen, C. J. Clinical Investigation of Improved Crystalline Insulin, Preliminary Report, *J A M A* 107 959, 1936.
7. Altshuler, S. S., and Leiser, R. Clinical Experience With Improved Crystalline Insulin, *J A M A* 107 1626, 1936.
8. Barach, J. H. Crystalline Insulin, *Ann Int Med* 10 1335, 1937.
9. Altshuler, S. S., and Leiser, R. Effect of Zinc Content Upon Action of Insulins, *Am J M Sc* 195 234, 1938.
10. Sayhun, M. Personal communication to authors.
11. Sindoni, A., Jr. Protamine Insulin Versus Ordinary Insulin, *J A M A* 108 1320, 1937.

CHANGES IN SPLEEN SIZE, BLOOD PRESSURE, AND ERYTHROCYTE  
COUNT AFTER THE ADMINISTRATION OF BENZEDRINE  
SULFATE (PHENYL-1, AMINO-2 PROPANE  
SULFATE) IN DOGS\*

J. L. PINKSTON, B.A., AND J. O. PINKSTON, PH.D.  
BEIRUT, LEBANON

THE response of the spleen to certain of the sympathomimetic amines has been observed by many investigators. Contraction of the spleen in response to epinephrine has been demonstrated by Hoskins and Gunning,<sup>1</sup> Tournade and Chabrol,<sup>2</sup> Barcroft,<sup>3</sup> and others. Izquierdo and Cannon<sup>4</sup> carried out a study on emotional polycythemia; they consider that splenic contraction may be responsible for an increase of up to about 20 per cent in the number of circulating erythrocytes, and that other factors enter into any greater increase.

The response of the spleen of the dog to ephedrine was briefly studied in 1930 by Marcou and Barcroft (see Barcroft<sup>3</sup>). They observed marked contraction, which passed off gradually during a period of about two hours. Davis,<sup>5</sup> using the technique devised by Barcroft and Stephens,<sup>6</sup> also showed contraction of the exteriorized spleen in response to ephedrine in dogs. Simpson and Cadness<sup>7</sup> found the increase in erythrocyte, leucocyte, and platelet counts in response to ephedrine essentially the same before and after splenectomy in the guinea pig, and they conclude: "The spleen is therefore not responsible for these changes in this animal."

Detrick, Millikan, Modern and Thienes,<sup>8</sup> in a study of the effects of benzedrine (amphetamine) on isolated smooth muscles, state in their summary: "In high concentrations ( $10^{-4}$  or higher) benzedrine caused contractions of all smooth muscles."

In a study of the functional and tissue changes following large doses of benzedrine sulfate (amphetamine sulfate) in the albino rat, Ehrlich and Krum-bhaar<sup>9</sup> found at autopsy that animals which died after having received 150 to 400 mg. of the drug showed contraction of the spleen; they attributed this contraction to the action of the benzedrine. These authors also found that in general an erythrocytosis occurred following benzedrine administration.

Myerson, Loman, and Dameshek<sup>10</sup> say concerning certain responses to benzedrine in man: "Its effects on the blood cellular constituents are probably due to vasoconstriction of the spleen and other reservoirs of blood cells resulting in an increased number of cells in the peripheral blood." Schube, Raskin, and Campbell<sup>11</sup> found no demonstrable permanent alteration of the cellular constituents or hemoglobin of the blood in human beings following the administration of benzedrine in dosage within the clinical range.

\*From the Department of Pharmacology, School of Medicine, American University of Beirut, Beirut, Lebanon. Aided by a grant from Smith, Kline and French Laboratories.  
Received for publication, December 7, 1938.

The present experiments were undertaken in order to investigate more fully the response of the spleen to the intravenous administration of benzedrine, and, in particular, to seek any correlation between splenic response and erythrocytosis occurring under the same circumstances.

Since the spleen of the dog contains sufficient smooth muscle to show visible changes in size when contraction occurs, photographs taken at stated intervals were used to record these changes. The position and adjustment of the camera remained constant throughout each experiment. This method of recording has obvious limitations, but serves to show gross alterations in size.

The experiments fall into two general groups—one in which we have sought to discover the possible correlation between changes in blood pressure and changes in spleen size, and another in which any correlation between changes in spleen size and erythrocyte count has been sought.

#### I. RESPONSES OF THE SPLEEN AND THE BLOOD PRESSURE

*Methods.*—A series of healthy male and female dogs was used, the average weight of dog being about 9 kg. They were anesthetized in respective cases with ether and chloralose, dial,\* and nembutal; the responses were essentially the same irrespective of the anesthetic used. Body temperature was maintained within the normal range by means of a heating pad and checked by a rectal thermometer kept in place throughout each experiment. The spleen was delivered through a left lateral incision with little laceration as possible, and then surrounded by towels kept moist with physiological saline. The splenic blood supply was carefully left unimpeded, and the organ was exposed only while being photographed. Very little blood was lost during the operative procedures. Blood pressure was recorded throughout each experiment by means of an ordinary mercury manometer. In all cases each injection of benzedrine consisted of 1 mg. per kg., a dosage chosen arbitrarily on the basis of its effectiveness and nontoxicity in the acute procedure.

Procedures of three types were carried out:

(a) Experiments (13) in which a first injection of benzedrine was given immediately after exposure of the spleen, and followed within one to two hours by a second injection.

(b) Experiments (5) in which a single injection was given immediately after exposure, and the effects followed for six to eight hours.

(c) Experiments (7) in which the spleen was exposed as described for six to eight hours, and an injection of the drug given only after this period of exposure.

*Results.*—(a) The first injection was followed within a few seconds by a rise in blood pressure of from 21 to 91 per cent (average, 48 per cent), which reached its height within about one and one-half minutes, and had returned to approximately its initial level within a maximum of fifteen minutes. Contraction of the spleen began while the pressor response was still fairly marked, and reached a maximum within about thirty minutes, long past the time when the pressor response had subsided. The second injection, given within one to two hours

\*Supplied by the Ciba Laboratories, Lyon, France.

(before there was perceptible relaxation of the spleen), was followed within a few minutes by a visibly greater contraction (Fig. 1, B and C). In a few cases there was a slight rise in blood pressure following the second injection, but the majority of cases showed either no change or a moderate fall of the blood pressure, which then gradually returned to the original level. This alteration in the blood pressure response to successive injections of benzedrine in the same animal has been observed by Tainter,<sup>13</sup> Detrick, Millikan, Modern, and Thienes,<sup>8</sup> and others.

The blood pressure level immediately before injection varied from 90 to 160, without appreciably affecting the nature or degree of response to the drug.

(b) Following a single injection immediately after exposure, the splenic contraction was observed to persist with but slight gradual relaxation for up to six hours (Fig. 2). The blood pressure response was as in (a).

(c) In the spleen exposed for six hours, slight gradual contraction occurred (Fig. 3, A, B, and C) without significant change in the blood pressure level during that period.\* At the end of six hours' exposure, an injection was followed within a few minutes by visible contraction (Fig. 3, D) and a pressor response of the type described in (a).

## II. RESPONSES OF THE SPLEEN AND THE ERYTHROCYTE COUNT

*Methods.*—A second, similar group of dogs was used. Dial anesthesia was employed throughout, and a single intravenous injection of warm physiologic saline was given soon after anesthetization. The amount, 25 to 75 c.c., was determined by the size of the animal, and at least twenty minutes was allowed to elapse before beginning blood counts.

Erythrocyte counts were made on venous blood at recorded intervals. Samples were obtained from alternate leg veins by means of a perfectly dry 2 c.c. syringe and a 25 gauge needle; care was taken that the limbs were warm and not constricted. About 0.5 c.c. of blood was withdrawn, placed on a paraffin-covered watch glass, and immediately taken up into a Trenner pipette for dilution with Hayem's fluid. Counts were made from a Neubauer chamber with a standardized cover slip; the same pipette and chamber were used throughout the series, and conditions and technique kept as uniform as possible. A sufficient number of counts was done in each case to check closely on technical accuracy.

In the cases indicated, the spleen was removed, and the abdomen was closed after complete hemostasis had been achieved.

Changes in spleen size were recorded as in I, but blood pressure was not recorded. Each injection of benzedrine consisted of 1 mg. per kg.

Procedures of three types were employed:

(a) Experiments (10) in which a series of counts were carried out in the anesthetized animal, and the spleen then exposed as described. After a second group of counts, the benzedrine was administered, and the erythrocyte count followed for at least one hour thereafter.

\*That the exposed spleen will shrink somewhat, and that after contraction it will not return to quite its original size while exposed, is to be expected, as has been demonstrated by Barcroft and his co-workers.

(b) Experiments (6) in which the same procedure was carried out, except that the spleen was removed rather than simply exposed

(c) Experiments (6) in which the adrenals were removed\* in a preliminary acute operation, and a group of counts were then taken. After establishment of the erythrocyte level, benzedrine was administered and the count followed as in (a) and (b)

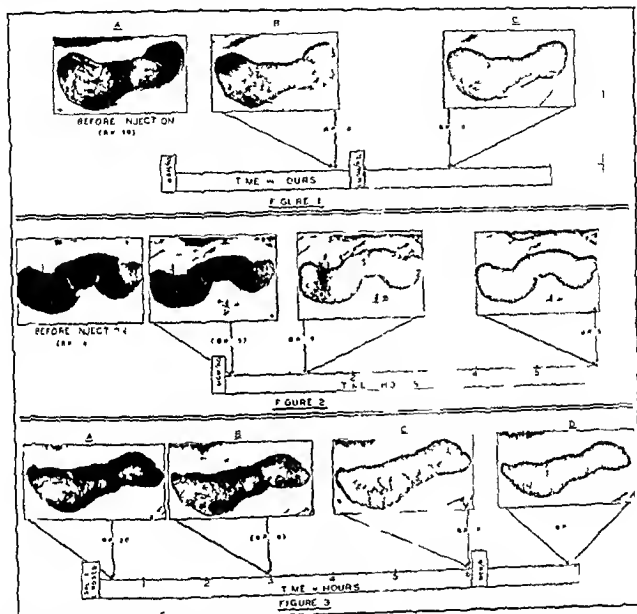


Fig 1—Changes of spleen size in the dog after the intravenous administration of two doses of 1 mg per kg of benzedrine, one given within ten minutes after exposure of the spleen, the second within one hour after the first

Fig 2—Changes of spleen size in the dog after the intravenous administration of a single 1 mg per kg dose of benzedrine within ten minutes after exposure of the spleen

Fig 3—A, B, and C—Changes of spleen size in the dog during six hours of simple exposure. D—Change following a single dose of 1 mg per kg of benzedrine given after six hours of exposure

**Results**—(a) The erythrocyte level was established by means of three or more counts before the administration of benzedrine in each case. Following the injection, there occurred a rise of from 2 to 29 per cent within twenty to seventy minutes, with an average rise of 16 per cent within an average time of thirty six minutes. The extreme figures of a 2 per cent rise in seventy minutes were obtained in a single experiment (5/23/38, Fig 5), the next lowest rise was 9 per cent, and the next longest time forty five minutes.

\*In one dog (11/2/38) Fig. 7 the adrenals were tied off. Autopsy was done at the conclusion of the experiment in order to be certain that the adrenals had been excluded from the circulation.



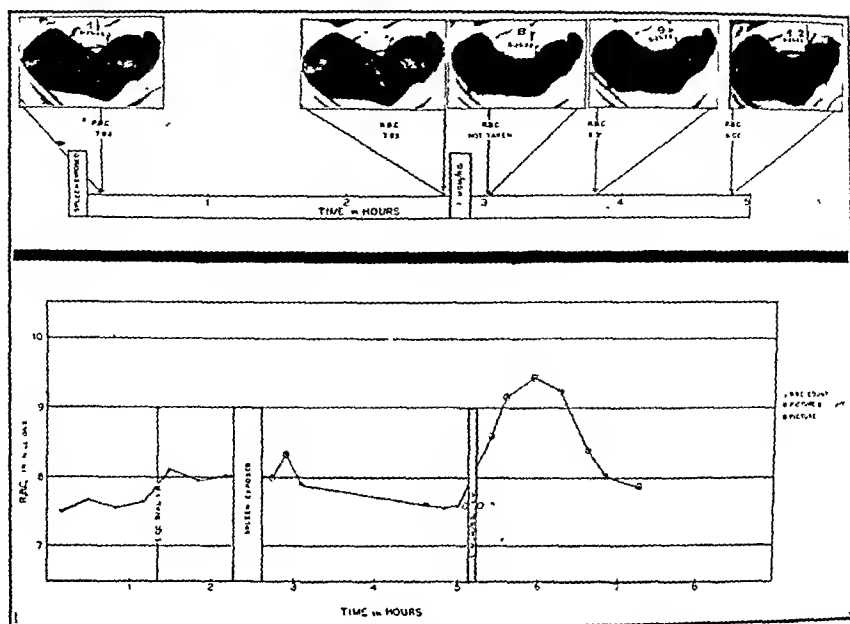


Fig. 4.—Changes of spleen size and erythrocyte count in the dog after exposure of the spleen and subsequent intravenous administration of 1 mg. per kg. of benzedrine.

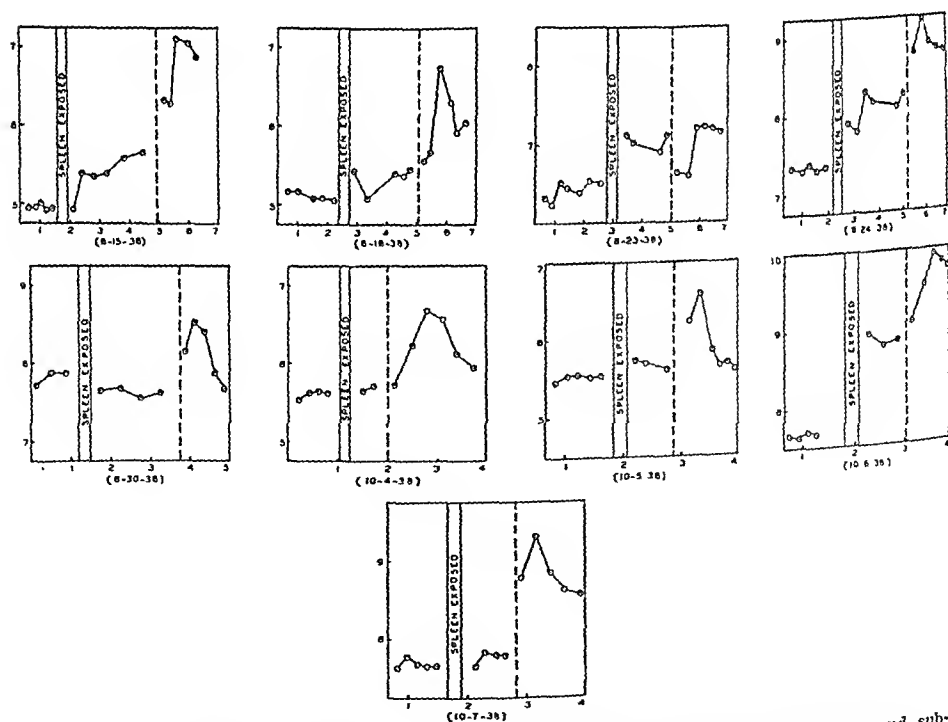


Fig. 5.—Changes of erythrocyte count in 9 dogs after exposure of the spleen and subsequent intravenous injection of 1 mg. per kg. of benzedrine (dotted line). Abscissae (scale value not the same in every case), time in hours; ordinates, erythrocyte count in millions.

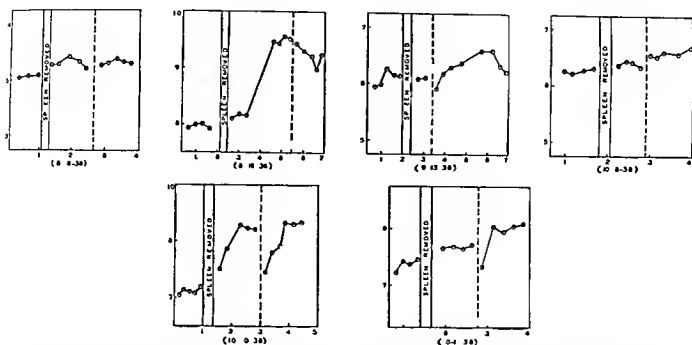


Fig 6—Changes of erythrocyte count in 6 dogs after removal of the spleen and subsequent intravenous injection of 1 mg per kg of benzedrine (dotted line) Abscissae (scale value not the same in every case), time in hours ordinates erythrocyte count in millions

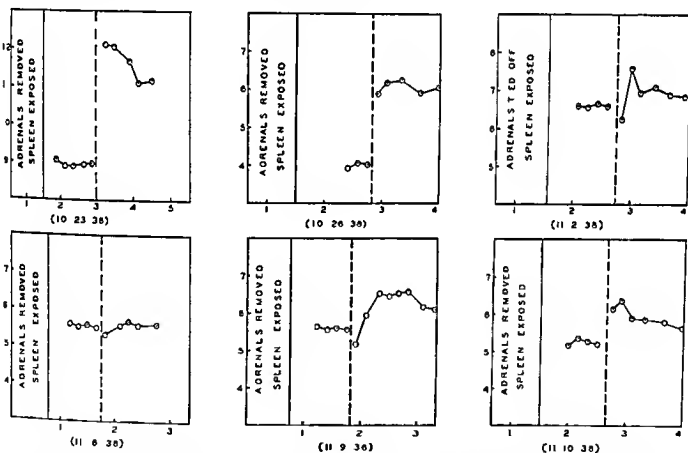


Fig 7—Changes of erythrocyte count in 6 dogs after removal of the adrenals with exposure of the spleen and subsequent intravenous injection of 1 mg per kg of benzedrine (dotted line) Abscissae (scale value not the same in every case) time in hours ordinates erythrocyte count in millions

A typical experiment of this type is illustrated in Fig 4, and the remainder of the group is represented graphically in Fig 5

(b) In those animals that underwent splenectomy, benzedrine administration was followed in some cases by an immediate, slight fall and then a very gradual, moderate rise of the blood count. In other cases, this rise occurred slowly without the preliminary fall. In no case did the rise of red blood cell count exceed 7 per cent in the period of from sixty to one hundred and eighty minutes during which the counts were followed after injection. A single case

showed a fall of 5 per cent within seventy minutes, and the count remained at 2 per cent below the initial level at the end of ninety minutes, when counts were discontinued.

This group is represented graphically in Fig. 6.

(c) In those cases in which the adrenals were removed (or tied off, as in one case), rises in erythrocyte counts were observed following the administration of benzedrine. The results were so variable, ranging from 2 to 55 per cent change within ten to sixty minutes (Fig. 7), that no exact conclusion concerning the role of the adrenals can be reached from this short series. It is clear, however, that the erythrocytosis observed after administration of benzedrine occurs in the adrenalectomized animal.

#### DISCUSSION AND CONCLUSIONS

The role of the spleen in the occurrence of erythrocytosis in dogs and cats following the administration of certain of the sympathomimetic amines and after sympathetic stimulation has been well established by investigators previously cited in this paper. In the present experiments, benzedrine sulfate was consistently found to cause contraction of the spleen, accompanied by promptly occurring erythrocytosis, after intravenous administration of 1 mg. per kg. in the dog. This erythrocytosis was likewise observed along with splenic contraction in adrenalectomized dogs, but did not occur in splenectomized dogs having intact adrenals.

These results, along with the extensive investigations of other workers, indicate that the spleen is the principal factor responsible for prompt and significant increases in the red blood cell count in the dog.

#### SUMMARY

1. In anesthetized dogs having the spleen exposed acutely, prolonged contraction of the spleen and a definite, transitory rise of blood pressure were observed following the intravenous administration of 1 mg. per kg. of benzedrine. A second injection of 1 mg. per kg. in the same animal was followed by further splenic contraction, but blood pressure showed either no change or a slight, transitory fall (13 experiments).

2. In anesthetized dogs having the spleen exposed acutely, contraction of the spleen following the intravenous administration of 1 mg. per kg. of benzedrine was observed to be accompanied by a rapidly occurring erythrocytosis of significant degree (10 experiments).

3. In anesthetized dogs having the spleen exposed and the adrenals removed acutely, responses of the spleen and the erythrocyte count after benzedrine administration were essentially the same as in 1 and 2 (6 experiments).

4. In anesthetized dogs having the spleen removed acutely, and the adrenals intact, no significant erythrocytosis occurred following the administration of benzedrine, except after a period of at least an hour, and probably not in direct response to the action of this drug (6 experiments).

## REFERENCES

- 1 Hoskins, R G, and Gunning, R E Lee The Affects of Adrenin on the Distribution of the Blood II Volume Changes and Venous Discharge in the Spleen, *Am J Physiol* 43 298, 1917
- 2 Tournade, A, and Chabrol, M La contraction de la rate par excitation du splinique releve d'un double mecanisme nerveux et adrenalinique, *Compt rend Soc de biol* 91 835, 1924 Secréction Reflexe d'adrenaline, *Ibid* 92 418 1925
- 3 Barcroft, I Some Effects of Emotion on the Volume of the Spleen, *J Physiol* 68 375, 1930
- 4 Izquierdo, J J, and Cannon, Walter B Studies on the Conditions of Activity in Endocrine Glands XVIII Emotional Polyethemia in Relation to Sympathetic and Medulladrenal Action on the Spleen *Am J Physiol* 84 545, 1928
- 5 Davis John E Response of the Exteriorized Spleen to Ephedrine, Acetyl Choline, Pilocarpine and Pituitrin (Posterior Pituitary Preparation), *Proc Soc Exper Biol & Med* 36 71, 1937
- 6 Barcroft, I, and Stephens, I G Some Observations Upon the Size of the Spleen, *J Physiol* 64 1, 1927
- 7 Simpson L, and Cadness, B H E Effects of Ephedrine on Erythrocytes, Leukocytes and Platelets in the Normal and Splenectomized Guinea Pig *J Pharmacol & Exper Therap* 56 389, 1936
- 8 Detrick, L E, Millikan, Ralph Modern F S and Thienes C H On the Pharmacology of Phenylisopropylamine (Benzedrine), *J Pharmacol & Exper Therap* 60 56, 1937
- 9 Ehrlich, W E, and Krumbhaar, E B The Effects of Large Doses of Benzedrine Sulfate on the Albino Rat Functional and Tissue Changes, *Ann Int Med* 10 1874, 1937
- 10 Myerson, A, Loman, J, and Dameshek W Physiologic Effects of Benzedrine and Its Relationship to Other Drugs Affecting the Autonomic Nervous System, *Am J M Sc* 192 560 1936
- 11 Schube, P G, Raskin Naomi, and Campbell Eleanor The Effect of Benzedrine Sulfate on the Hemopoietic System, *New England J Med* 216 922, 1937
- 12 Tunker, M L Comparative Actions of Sympathomimetic Compounds Phenyl and Substituted Phenyl Derivatives Non Phenolic Ring Compounds and Aliphatic Amines, *Arch internat de pharmacodyn et de therap* 46 192 1933

## CHYLOTHORAX AND CHYLOUS ASCITES\*

## REPORT OF A CASE WITH LIPID ANALYSIS

GEORGE T HARRELL, M D, DANA M STREET, M D, AND  
RAYMOND REISER, PH D, DURHAM N C

THE accumulation of chylous fluid in the serous cavities of the body is an event of relatively rare occurrence Bauersfeld<sup>1</sup> noted 49 cases of traumatic chylothorax reported in the literature Yater<sup>2</sup> found less than 100 cases of non-traumatic chylothorax, and only 3 of chylopericardium Chylous ascites may accompany chylothorax, or it may occur alone The commoner causes of chylous effusion are trauma, invasion, compression, or occlusion of the thoracic duct by a malignant tumor or leucemia, thrombosis of the great veins at the exit of the duct, or infection, usually tuberculous, with obstruction of the duct and smaller lymphatics

Lee<sup>3</sup> has experimentally shown that chylous fluid rarely forms from occlusion of the duct alone The extent of the lymphatic supply, the patency of collaterals,

\*From the Departments of Medicine and Pathology Duke University School of Medicine Durham

the level and completeness of the obstruction are other factors. Attempts have been made to classify the fluids as chylous, chyloform, or pseudochylous by their appearance and fat content.<sup>3</sup> These groups are not distinct, for the appearance and composition are dependent on the size of the fat particles, the relative proportion of lymph and chyle, the cellular content, the diet, and the lipid partition. Lactescent fluids are also said to occur from degeneration of the cells in exudates and from dispersion of such substances as "glycoprotein" or lecithin-globulin complex.

The reports in the earlier literature of the chemical composition of these fluids have been reviewed by Gandin.<sup>3</sup> Since the introduction of more modern and accurate methods of fat analysis, determinations on human material have been rare. Robinson and his co-workers<sup>6</sup> have analyzed fluids experimentally produced in cats and dogs. Since none of the previous reports have compared the fluid in the various cavities, it was thought the studies upon the following case warranted recording.

#### CASE REPORT

A 49-year-old white tenant farmer was first admitted January 31, 1937, complaining of the loss of 45 pounds in weight in the preceding three months. For five years the patient had noted some burning in the epigastrium with fullness after meals. For three months he had been vomiting shortly after meals once or twice daily. He had taken little by mouth for six days.

On admission the temperature was 36.5° C., pulse 100, respirations 20, and blood pressure 125/85. The patient was weak, chronically ill, and showed evidence of weight loss. A smooth, firm, nontender mass was present in the epigastrium, and extended 4 cm. below the xiphoid process. The remainder of the physical examination revealed no abnormal findings. Wassermann and Kahn tests were negative. The blood sugar on admission was 880 mg. per cent; chlorides, 320 mg. per cent; carbon dioxide combining power, 96 volumes per cent; nonprotein nitrogen, 125 mg. per cent; cholesterol, 128 mg. per cent. Stools were negative for occult blood. X-rays of the gastrointestinal tract showed only a high hypertonic stomach with thickened rugae.

After an afebrile course of nineteen days on a diabetic regime, he was discharged symptom free. During the next two months his insulin requirement steadily reduced.

He was re-admitted on April 24, 1937, three months after his first admission, complaining of an upper respiratory infection of eighteen days' duration, associated with abdominal pain and vomiting. Five days previously he had noticed a swelling in the left side of his neck. At this time the temperature was 36.5° C., pulse 140, respirations 22, blood pressure 114/80. The positive physical findings were limited to small palpable lymph nodes in the neck, an indefinite mass in the region of the left sternocleidomastoid muscle, moist râles at the lung bases, and absent knee jerks. No mass was palpable in the epigastrium, and there was no jaundice.

The red blood cell count was 3,800,000, the white blood cells totaled 16,600, with 96 per cent polymorphonuclear leucocytes. The blood chemistry findings were within normal limits. Gastrointestinal x-rays demonstrated a triangular notch on the greater curvature of the stomach, and delay in emptying of the duodenum with the second and third portions fixed and rigid. This was interpreted as extrinsic involvement by tumor. On admission chest plates revealed prominent hilar shadows, but no fluid. Metastatic carcinoma was found in microscopic sections of a lymph node removed from the neck.

Fluid rapidly accumulated in both sides of the chest and in the abdomen. New tumor nodules appeared in the left side of the neck, with edema of the right side of the face and the left hand. The mucous membranes became cyanotic. He died quietly on his eighteenth day in the hospital.

*Autopsy*—Autopsy was performed thirty minutes after death. Externally the cervical and left axillary lymph nodes were enlarged. Two and eight tenths liters of grayish yellow chylous fluid, with an odor resembling that of mull, were removed from the peritoneal cavity, and one liter of similar fluid from each of the pleural cavities. The pericardium contained 20 cc of clear straw colored fluid. The peritoneal surfaces were smooth glistening, and free, except for old fibrous adhesions between the gall bladder, duodenum, and transverse colon. The lymphatics in the serosa of the entire intestine were distended with chyle, resembling the lacteals of experimental animals fed on fat (Fig 1).

The hilar lymph nodes were greatly enlarged, up to 3 cm in diameter, and contained pockets of creamy material, 3 mm in size which could be expressed from the sinuses. A gray tumor nodule, 1 by 0.5 cm in size, was found in a large vein of the posterior portion of the right lung. No tumor nodules were found in the lung parenchyma or bronchi.



Fig 1—Loop of small intestine showing lacteals filled with chyle

The liver weighed 1940 gm, small, round, firm, white nodules 2 mm in size, were scattered throughout. No cirrhosis was present. The bile ducts and portal vein at the hilum of the liver were surrounded by a 2 cm mass of tumor tissue, but the ducts were not obstructed. The pancreas, yellowish brown in color, weighed 160 gm and was densely embedded in fibrous tissue. The head was enlarged, surrounded by nodules of tumor tissue, and contained a single small nodule 3 cm in size. The soft spleen weighed 150 gm, the splenic vein contained tumor tissue. The adrenals weighed 40 gm together the tissue was almost entirely replaced by tumor nodules of 1 cm size or less.

On the greater curvature of the stomach was an old ulcer, perforation of which had been closed by adhesion between the stomach wall, the lower margin of the pancreas, and the posterior abdominal wall. The walls of the second and third portions of the duodenum were thickened and surrounded by dense fibrous tissue, the lumen was narrowed.

The thoracic duct was enlarged and patent to a point above the arch of the aorta, where it was buried in a mass of supraclavicular nodes, 6 cm in diameter. No lymphatic collaterals, right thoracic, or accessory ducts were seen, though dye was injected into the thoracic duct. No thrombus was noted in the great veins of the neck or thorax.

In microscopic preparations of the ulcer in the stomach large, eosinophilic, vacuolated cells, with eccentrically placed nuclei, were found at the edges, infiltrating the wall, suggest

ing the origin of the tumor from mucous cells. No glandular patterns or areas of colloid were found. At numerous other places throughout the body, the tumor cells were anaplastic and mitoses were numerous. Dilated lymphatics, filled with eosinophilic material staining brightly with scharlach R and containing strands and sheets of tumor cells, were found in the mucosa of the duodenum and gall bladder, fibrous tissue septa of the pancreas, and other viscera. In lymph nodes, the tumor showed necrosis. The branches of the thoracic duct, which was multiple near its termination, were almost filled with tumor cells (Fig. 2). Although the primary site was probably in the stomach, the pancreas, gall bladder, and duodenum could not be definitely excluded.

*Analysis of Fluids.*—The characteristics of the fluids aspirated are given in Table I. All were sterile on culture and contained small numbers of large oval cells, twice the size of lymphocytes, which were thought to be tumor cells. Fat droplets of varying size and number were stained by sudan III.

TABLE I

	RIGHT PLEURA		LEFT PLEURA	PERITONEUM
Date	5/1/37	5/5/37	5/5/37	5/3/38
Quantity	450 c.c.	-----	130 c.c.	4 liters
Character	opalescent	opalescent	turbid	creamy
Color	yellow	greenish yellow	chylous	-----
Specific gravity	1.016	-----	-----	1.022
W.B.C.	125/mm <sup>3</sup>	450/mm <sup>3</sup>	315/mm <sup>3</sup>	400/mm <sup>3</sup>
R.B.C.	75/mm <sup>3</sup>	280/mm <sup>3</sup>	90/mm <sup>3</sup>	100/3
Protein*	3.5 gm. %	-----	-----	4.0 gm. %

\*Esbach tube method.

The lipid analyses were done by a modification of the method previously reported by one of us.<sup>5</sup> The phospholipids were determined according to Boyd<sup>2</sup> and the free cholesterol on the acetone washings. The bound cholesterol digitonide was determined on the washings from the free cholesterol and the neutral fat fatty acids on the washings from the bound. The results on fluid aspirated six to eight days before death are given in Table II. The diet contained 120 gm. of fat daily.

TABLE II

	RIGHT PLEURA	LEFT PLEURA	PERITONEUM
	mg. %	mg. %	mg. %
Phospholipid	50	73	105
Free cholesterol	26	41	48
Cholesterol as esters	39	54	64
Neutral fat fatty acids	102	271	604
Total lipid	217	439	821

## COMMENT

Leucemias and neoplasms are the most common etiologic agents in non-traumatic chylous effusion. Of the malignancies, carcinoma of the stomach is the most frequent. Robinson<sup>6</sup> showed that ligation of the superior vena cava alone could produce chylous effusions, though ligation of both the right and left thoracic ducts would not. No thrombus or obstruction was found at this site in the present case. The anastomatic collateral lymphatic circulation and lymphaticovenous connections, which Lee<sup>4</sup> described as developing after ligation of the thoracic duct, prevent this accumulation of fluid. Though the larger collaterals were not demonstrated, the main branches of the thoracic duct (Fig. 2), and many of the smaller lymphatics (Fig. 3) in the abdominal organs were found permeated and partially obstructed by masses of degenerating tumor cells. This is said to result in an elevated pressure in the lacteals, with increased



Fig 2—Thoracic duct near its termination showing multiple channels with masses of tumor cells and old thrombi in the lumina ( $\times 25$ )

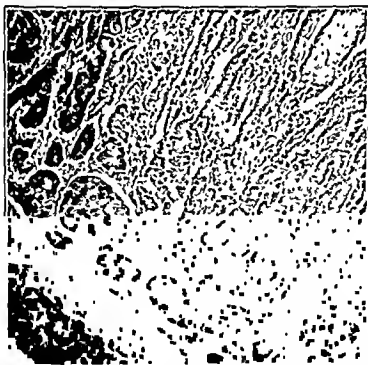


Fig 3—Stomach showing carcinoma cells blocking small lymphatics at the muscularis mucosae ( $\times 110$ )

transudation of the smaller fat particles through the intact walls, as well as rupture of the weaker ones and direct spilling of chyle into the free peritoneum. Such breaks are rarely proved. We can only speculate as to the cause of the variation in appearance and fat content in the various cavities. In traumatic chylothorax, the effusion occurs more often on the right, because of the anatomic arrangement of the duct.<sup>1</sup> In the present instance the most likely explanation is that the collaterals to the right thoracic duct, or the lymphatico-venous connections to the azygos vein, were the last to be occluded. That the



process was a progressive one is indicated by the finding at autopsy of frankly chylous fluid in both pleural cavities.

#### SUMMARY

A case of bilateral chylothorax with chylous ascites as the result of obstruction to lymphatics and the thoracic duct by carcinoma cells from a primary tumor of the stomach is reported.

Lipid analyses on fluid removed during life with the patient on a controlled fat intake show increasing fat content, chiefly in the neutral fat fatty acid fraction, in the right and left pleural and peritoneal cavities, respectively.

#### REFERENCES

1. Bauersfeld, E. H.: Traumatic Chylothorax From Ruptured Thoracic Duct, *J. A. M. A.* 109: 16, 1937.
2. Boyd, E. M.: Low Phospholipid Values in Dog Plasma, *J. Biol. Chem.* 91: 1, 1931.
3. Gandin, S. V.: Pathogenese und Klassifikation der milchartigen Ergüsse, *Ergebn. d. inn. Med. v. Kinderh.* 12: 218, 1913.
4. Lee, F. C.: The Establishment of Collateral Circulation Following Ligation of the Thoracic Duct, *Bull. Johns Hopkins Hosp.* 33: 21, 1922.
5. Reiser, R.: Lipid Analysis of Human Thoracic Duct Lymph, *J. Biol. Chem.* 120: 625, 1937.
6. Robinson, C. S., Cunningham, R. S., Blalock, A., Gray, M. E., and Rogers, B. C.: Chylous Effusions Produced by Experimental Ligation of the Superior Vena Cava. Chemical and Cytologic Studies, *Arch. Path.* 24: 303, 1937.
7. Yater, W. M.: Non-traumatic Chylothorax and Chylopericardium: Review and Report of a Case Due to Carcinomatous Thromboangiitis Obliterans of the Thoracic Duct and Upper Great Veins, *Ann. Int. Med.* 9: 600, 1935.

---

## HYPERINSULINISM DUE TO ADENOMA OF THE PANCREAS

---

### REPORT OF A CASE

ROBERT G. MURPHY, M.D., CECIL C. DUSTIN, M.D., AND  
RUSSEL O. BOWMAN, PH.D., PROVIDENCE, R. I.

**A** CASE history of a man who suffered from hyperinsulinism due to adenoma of the pancreas is presented.

Seale Harris<sup>1</sup> in 1924 first described the condition of hyperinsulinism due to excessive secretion of insulin. This has been shown to have a great variety of manifestations, such as low blood sugar, hunger, sweating, nervousness, weakness, tremors, mental lapses, convulsions, delirium, and coma.

Numerous cases of tumors of the pancreas associated with the symptoms of hyperinsulinism have been reported. The first case proved pathologically was described by Wilder, Allen, Power, and Robertson<sup>2</sup> in 1927. It occurred in a 40-year-old physician who had attacks of unconsciousness with convulsions due to a carcinoma of the pancreas. An extract of one of the liver metastases acted like insulin when injected into rabbits; this proved the tumor cells capable of producing insulin.

In 1926 Warren<sup>3</sup> reported 4 cases and collected 16 others from the literature

Since Warren's paper other reports of hyperinsulinism have appeared.<sup>4</sup> These have been associated with carcinoma of islet cells, adenoma of the pancreas, or hyperplasia of the islet tissue. Reports have appeared in which there was hyperfunction of a morphologically normal pancreas, and a recent report claims relief of the hypoglycemia attacks after removal of a calcified tumor from the head of the pancreas.<sup>5</sup> Relief of hypoglycemic symptoms by appropriate surgical procedures, including removal of tumors and subtotal resection of the pancreas, has also been reported.

#### CASE REPORT

J. C., an Irish male, aged 60 years, was admitted to the Medical Service of Rhode Island Hospital after having been confused and disoriented for three hours. For a period of seven months previous to admission, he had had similar attacks about twice weekly. On these occasions, usually just before the evening meal the patient became delirious, crossed and uncrossed his legs, and waved his arms about his head occasionally falling. These attacks had been ascribed to alcoholism, though an alcoholic breath was never noted. There was no history of loss of consciousness, hunger pangs, weakness, sweating, palpitation, precordial pain, or dyspnea. It had not been noted that food relieved attacks. He was said to have been unusually irritable, and experienced occasional tremors which did not precede attacks. At times his gait was ataxic.

Physical examination revealed a well developed and well nourished man lying in bed, confused and disoriented, but with no other complaint. He was unable to recognize common objects and was reluctant to answer questions. His temperature was 98° F, his pulse rate was 67, and his respiratory rate was 20. There was enlargement of the heart to the left, a systolic murmur over the precordium, loudest at the apex, and marked sclerosis of the radial arteries. His blood pressure was 172 systolic and 95 diastolic. The knee jerks, biceps, and triceps tendon reflexes were hyperactive on the right and a doubtful Babinski was obtained on the right. Ophthalmoscopic examination of the optic fundi showed evidence of arteriosclerosis of the retinal arteries. There was no evidence of increased intracranial pressure.

Blood taken the morning after admission when the patient was fasting showed 43 mg of glucose per 100 cc, blood urea nitrogen of 11 mg per 100 cc, hemoglobin of 90 per cent (130 gm per 100 cc), and white blood cell count of 7550. Urine was slightly acid, had a specific gravity of 1.010, and was negative for sugar, albumin and sediment. An electrocardiogram was not significant. Because of the low fasting blood sugar, the possibility of an islet cell tumor of the pancreas was considered. Further work was done to confirm the diagnosis and to rule out other possible causes of a hypoglycemia.

A phenolsulfonphthalein test showed 55 per cent excretion in two hours after intramuscular injection of the dye. Blood Wassermann and Hinton tests were negative. On subsequent days the following findings, exclusive of blood sugars, were obtained: carbon dioxide combining power, 54 volumes per 100 cc of plasma, serum calcium 10.2 mg per 100 cc, serum inorganic phosphorus, 3.4 mg per 100 cc, total serum protein, 6.6 gm per 100 cc, serum potassium, 19.5 mg per 100 cc, whole blood sodium chloride, 388 mg per 100 cc, serum cholesterol, 110 mg per 100 cc, diastatic activity, 20 units (picric acid method), basal metabolic rate, minus 10.7 per cent. X-ray examination of the chest, examination of the gastrointestinal tract for duodenal deformity, and of the skull with special reference to the sella turcica, were all negative.

In Fig 1 are given the fasting blood sugar values obtained from the patient during his stay in the hospital. The Benedict<sup>6</sup> method, very nearly specific for glucose and monosaccharides of like reducing properties was used for blood sugar determinations. The patient had a blood sugar of 33 by the Benedict method, and of 43 by the Benedict Lewis picric acid method.

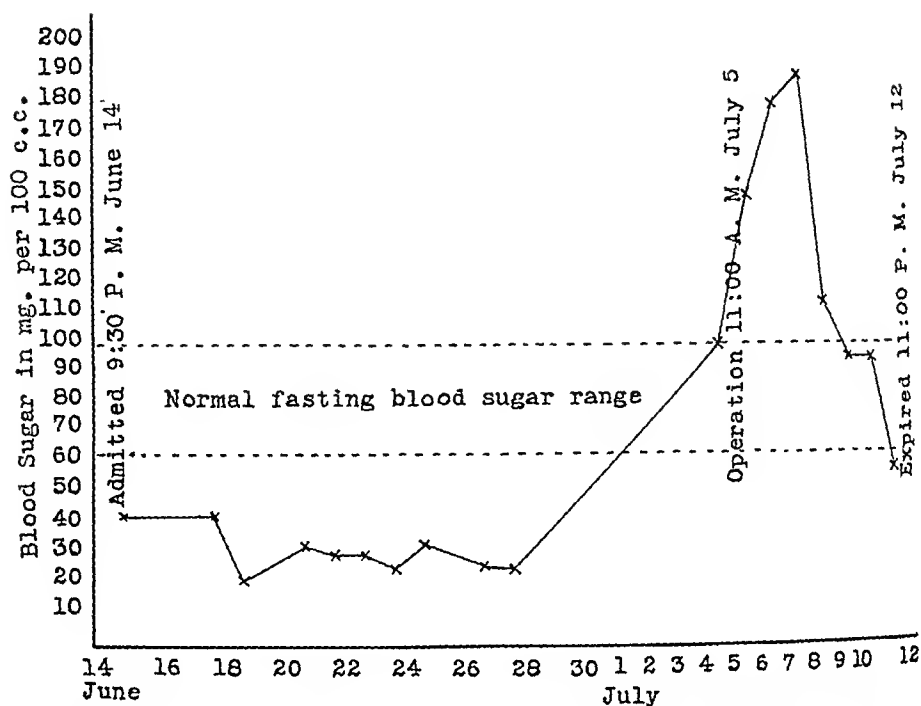


Fig. 1.—Fasting blood sugar values obtained on patient before and after operation.

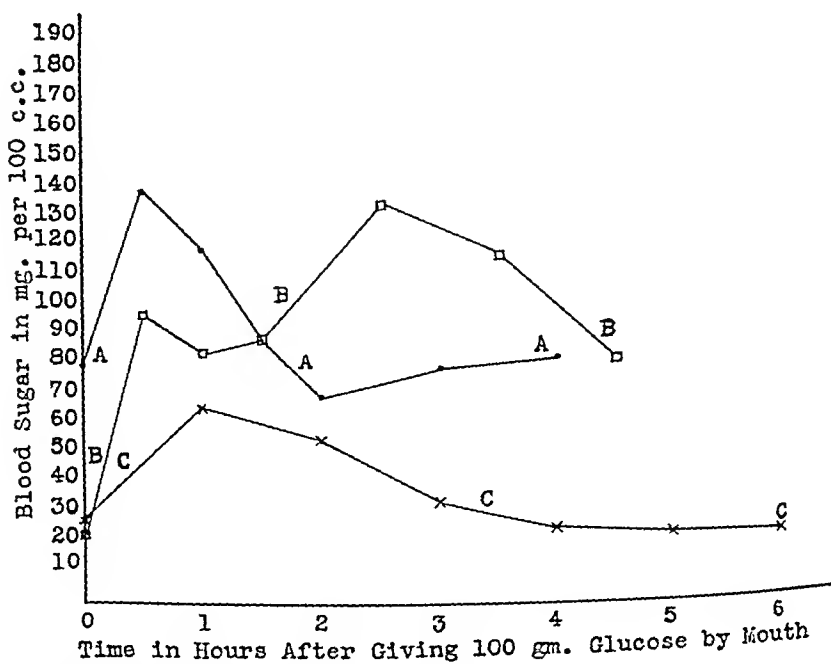


Fig. 2.—Glucose tolerance blood sugar curves.

- A Average normal curve.
- B Patient on 6-19 after house diet.
- ×—× C Patient on 6-27 after high carbohydrate diet.

Two sugar tolerance tests, with an average normal curve, are given in Fig 2. Curve B shows a maximum blood sugar response of 136 mg per 100 cc, reaching a maximum later than normal and returning toward the fasting value only after two and one half hours. Previous to this the patient had been on house diet. There is evidence from curve B that storage of sugar as glycogen occurs slowly. For three days previous to the second tolerance test, curve C, the patient had been on a 200 gm carbohydrate, 50 gm fat and 70 gm protein diet. A marked change in the type of curve is noted. The maximum value is less than half the former, and the return toward the fasting level is more prompt. Although the final values of this curve are 22 and 21 mg per 100 cc, the patient had no symptoms or physical signs of an impending attack. The difference between curves C and B was probably due to training of the sugar storing mechanism by a high carbohydrate diet previous to curve C.

Hyperinsulinism due to carcinoma, adenoma, or hyperplasia of the islet tissue of the pancreas was considered the probable diagnosis. Other causes for hypoglycemia, such as hypofunction of endocrine glands other than the pancreas (pituitary, adrenal thyroid, gonads), liver disease, muscular dystrophies, severe infections or prolonged starvation or fatigue, seemed to be excluded by laboratory tests, x ray data, and physical examination.

An attempt was made to decrease the response of the pancreas by giving 10 units of regular insulin one half hour after each meal, and a diet of 120 gm carbohydrate, 80 gm protein, and 188 gm fat. At 9:30 P.M. the third day of this regime the patient suddenly began to cross and uncross his legs, wave his arms and cry "Stop stop!" He was conscious but disoriented and confused. Each attempt to elicit a Babinski reflex stimulated the patient to greater activity and appeared to increase the severity of the attack. He was given 4 ounces of orange juice, and within five minutes became oriented and less confused, movement of his extremities ceased and could not be elicited by testing for the Babinski reflex. At 10:45 P.M. another attack of less severity occurred but it was again controlled by giving orange juice.

The diet was changed to one of 200 gm carbohydrate 50 gm fat and 70 gm protein.

A few days later a deliberate attempt was made to precipitate an attack by withholding the patient's breakfast. At 11:00 A.M. he showed marked tremors of the extremities, a fixed stare, profuse sweating, and pallor. He could not answer questions and became very restless, thrashed about in bed, and was restrained with difficulty. A blood sugar taken at this time showed 26 mg per 100 cc. Within three minutes after drinking 4 ounces of orange juice, the patient stopped thrashing about, answered questions and he felt fine, and did not remember what had just happened.

He was transferred to the Surgical Service for laparotomy and exploration of the pancreas. He was given intravenous injection of 500 cc of physiologic saline and 100 cc of 50 per cent glucose twice the day before operation and once just prior to operation.

Under gas oxygen ether anesthesia a left upper rectus incision was made. The gastrocolic omentum was divided, the pancreas was exposed, and a small tumor was palpated in the head of the gland. This tumor was shelled out by blunt dissection.

Following the operation, the patient was given intravenous glucose and saline daily. His fasting blood sugars, as seen in Fig 1, ranged from 100 the morning of operation to as high as 190 three days later, with gradual decline thereafter.

Urine sugars taken fasting were 1+ to 4+ for three days, and then became negative. Two days after operation his temperature rose to 101.8° F, his pulse rate to 126, and his respiration rate to 30. Examination of his chest showed signs of a bilateral bronchopneumonia which was considered to be a postoperative complication. His condition became progressively worse, and he died on the seventh postoperative day. During the postoperative course there were no symptoms or signs of hypoglycemia at any time. Permission for autopsy could not be obtained.

We are indebted to Dr. B. Earl Clarke, pathologist, for the photomicrograph (Fig 3) and for the following report of the tumor removed at operation:

*"Gross Description"* The specimen consists of a rather spherical tumor mass which measures approximately 1 cm in diameter. Part of its external surface is smooth and shiny as though it had been shelled from a capsule. However, elsewhere there are tags of tissue suggesting that it was adherent. The cut surface is rather grayish.

*“Microscopical Description:* There is an abundant dense hyaline stroma through which are scattered small areas of epithelial tissue, which vary in histology. There are several small ducts. There are also bits of glandular tissue like that of the external secretory part of the pancreas. There are, in addition, relatively large areas made up of narrow cords of cells in a close apposition to capillary blood vessels. These closely resemble normal islands.

*“Diagnosis:* Adenoma of the pancreas.”

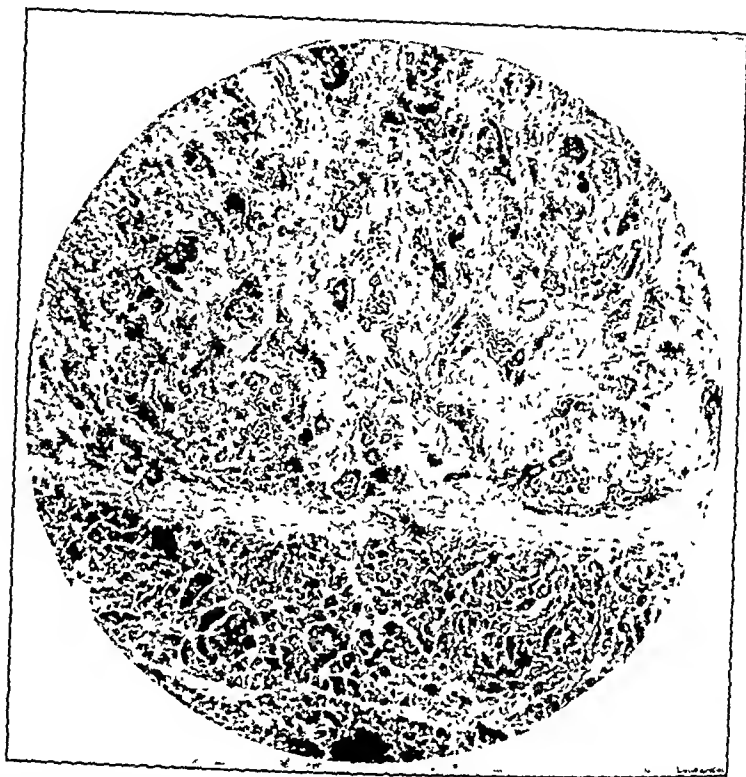


Fig. 3.—Low power photomicrograph showing the tumor (above) separated from the normal pancreatic tissue (below) by a distinct fibrous capsule. Note the abundant stroma and the variation of epithelial histology as described in the text. Hematoxylin and eosin preparation.

#### SUMMARY

A case of hyperinsulinism due to adenoma of the pancreas is presented with a description of hypoglycemic attacks and of the tumor removed by operation.

#### REFERENCES

1. Harris, S.: Hyperinsulinism and Dysinsulinism, *J. A. M. A.* 83: 729, 1924.
2. Wilder, R. M., Allen, F. N., Power, M. H., and Robertson, H. E.: Carcinoma of the Islands of the Pancreas: Hyperinsulinism and Hypoglycemia, *J. A. M. A.* 89: 348, 1927.
3. Warren, S.: Adenomas of the Islands of Langerhans, *Am. J. Path.* 2: 335, 1926.
4. A complete bibliography has been omitted for the sake of brevity. See Whipple, A. O.: *J. Internat. de Chir.* 3: 1, 1933.
5. Hermann, S. F., and Gius, J. A.: Hyperinsulinism Associated With Calcified Tumor of the Pancreas, *J. A. M. A.* 108: 1402, 1937.
6. Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and Saccharoids (Non-fermentable, Carbohydrate Reducing Substances), *J. Biol. Chem.* 92: 141, 1931.

# THE PHOTOCOLORIMETRIC DETERMINATION OF VITAMIN A AND CAROTENE IN HUMAN PLASMA\*

MARIAN STARK KIMBLE, PH D, MADISON, WIS

THE chief difficulty that has stood in the way of accurate quantitation of the Carr-Price reaction for determination of vitamin A is the very transitory character of the blue color that is produced. This comes to its height and begins to fade within a few seconds, and while the fading can apparently be somewhat retarded by a number of scrupulous precautions in the repurification and handling of the reagents, the necessity of their application so complicates the procedure as to restrict its practicability.

It is possible with the use of the Evelyn photoelectric colorimeter<sup>1</sup> to carry out the color producing reaction with the cell in the reading position, and by watching the swing of the galvanometer, to follow the evanescent color to its definite point of maximum. In a recent communication<sup>2</sup> Dann and Evelyn have described the use of this instrument in a method of precision for the assay of vitamin A in oils. Dr Charles May<sup>3†</sup> has developed a modification of the method for the determination of vitamin A in serum using the *micro* unit of the colorimeter,<sup>4</sup> which is desirable if very small quantities of blood must be used. Because of the advantages afforded by the *micro* unit in convenience, speed, and accuracy of operation, it was felt that a more direct adaptation of the technique of Dann and Evelyn to blood analysis would be justified and would permit its wider application and study. Such an adaptation has been used in the orientation studies which follow.

*Principle of the Method*—Blood plasma is extracted with alcohol and petroleum ether. The pigments dissolve in the alcohol, and carotene and vitamin A go quantitatively into the benzene layer. The yellow color of the carotene in the petroleum ether is read with filter 440 (blue) in the colorimeter as the basis for later correction of the blue color of the Carr-Price reaction, to which both carotene and vitamin A contribute. For the Carr-Price reaction the petroleum ether is aerated off and the residue is taken up in chloroform. Filter 620 (red) is used in the reading of the blue color. With the test tube cell containing the plasma extract in the reading position, the antimony trichloride reagent is added all at once, and the reading of the galvanometer recorded at the point of momentary stability which occurs at the height of color formation. After this, a reversal of the galvanometer swing indicates that fading has started. Since the rapidity of fading, rather than the point of color maximum, appears to be altered by ordinary impurities in the reagents, particular care in their repurification and anaerobic handling has not been found necessary with the use of the microinstrument.

\*From the Biochemical Laboratory, Department of Medicine, University of Wisconsin and State of Wisconsin General Hospital, Madison.

Received for publication December 30, 1933.

†Dr May has kindly furnished a manuscript copy of his procedure in the micromethod. His results have not been available up to this time of writing.

## METHOD

*Reagents.*—

1. 95 per cent alcohol.

2. Light petroleum ether. Mallinckrodt's ether, B.P. not over 80° C. was redistilled, and the fraction boiling under 45° used in all of the earlier analyses here reported. Later this was replaced by Baker's C.P. special benzine, B.P. 20°-40° C.

3. Chloroform. Anesthetic or U.S.P. XI chloroform has been used without further drying or purification. Exposure to air has been kept at a minimum by opening quantities which last only for a short time.

4. Antimony trichloride reagent: 25 per cent in chloroform, weight in volume. Solution without heating has been obtained by pulverizing the chemical before weighing and then crushing it further with a footed glass rod in a wide-mouthed glass-stoppered brown bottle in which it is both weighed and stored. The reagent is kept at room temperature, to avoid precipitation and irregular changes in concentration. Both Merck's and Mallinckrodt's analytical grades have been used interchangeably. *Precaution:* Rinse all utensils which have contained antimony trichloride with fairly strong hydrochloric acid before washing, since water decomposes the salt with the formation of slimy, tenacious precipitates.

*Colorimeter.*—Evelyn photoelectric macrocolorimeter with filters 440 and 620.\* A set of the specially selected ("S") absorption test tubes is reselected and marked to give uniform blank readings.

## PROCEDURE

Three and one-half to 5.0 c.c. (preferably 5.0) of plasma from oxalated blood (one drop of 30 per cent potassium oxalate to 10-12 c.c. of blood) are used for a determination. The sample is measured into a narrow-necked, glass-stoppered 25 c.c. centrifuge tube† and an equal volume of 95 per cent alcohol and 12 c.c. of petroleum ether, accurately measured, are added. The stopper is sealed in place with a small drop of mineral oil. The contents are mixed by end-over-end inversion of the tube for ten minutes, and then centrifuged for a short time to aid in layering. The tubes have regularly withstood speeds of 1700-2200 r.p.m. Ten cubic centimeters of the supernatant petroleum ether extract are pipetted into a colorimeter tube and the yellow color read with filter 440 against a petroleum ether blank. The galvanometer reading for carotene is recorded as  $G_{440}$ , and the corresponding optical density (read from the L-G table accompanying the instrument) as  $L_{440}$ . The petroleum ether is then evaporated off in a current of warm dry air or carbon dioxide.‡ For petroleum ether boiling

\*The colorimeter with necessary filters may be purchased from the Rubicon Company, 29 North Sixth Street, Philadelphia.

†Glass-stoppered centrifuge tubes of this kind have been obtained on special order through Elmer and Amend Company, specifying tubes similar to their catalogue No. 20061/6, but of 25 c.c. capacity.

‡Tanks of carbon dioxide with pressures too low to be used in the freezing of pathologic sections may be used for this purpose conveniently and without extra expense. Hence carbon dioxide has been used throughout most of the present work, though with determinations of blood A, aeration with room air proved to be equally satisfactory from the standpoint of recovery (see Table IV, Experiments 7 to 10). The carbon dioxide or air is delivered to a manifold through a bottle of calcium chloride.

under 45°, the 10 c.c. can be taken to dryness in about ten minutes with a stream of 6.7 L/minute, the tubes in a water bath at 40° to 43° C.

The outsides of the tubes are rinsed and dried, and the residue in each dissolved in 1 c.c. of chloroform. An antimony trichloride blank is set at 100 in the colorimeter with filter 620, and the center setting determined and allowed to become absolutely stable. The unknown tube with the chloroform extract is then put in place in the instrument, and 9 c.c. of antimony trichloride reagent is added rapidly down the side of the tube from a quick delivery pipette. (For this purpose a 10 c.c. transfer pipette may be cut off from the end and recalibrated.) The galvanometer will oscillate briefly, as mixing occurs and then move to the left to indicate a point of equilibrium which is transitory but unmistakable. Then it will reverse its direction and slowly move off again to the right as the color begins to fade. The galvanometer reading at the maximum color is recorded as  $G_{\max}$ , and its corresponding density as  $L$ . The maximum may persist for several seconds or for only one to two seconds depending apparently mostly upon the freshness of the reagent. It is important to note as pointed out by Dann and Evelyn,<sup>2</sup> that the *initial reading* when actually obtained at maximum color, is the same with old or new reagents although the *rate of fading* of the color may vary. This fact was borne out in the present study by comparisons of different reagents, to be discussed below.

#### CALCULATION OF RESULTS

The basis for the use of standard spectrophotometric formulas in the calculation of the results obtained with the photoelectric colorimeter—in effect an abridged spectrophotometer—is outlined by Dann and Evelyn in their discussion of the determination of vitamin A in oils.

When the initial light intensity affecting the photocell is adjusted to give a full scale deflection of 100 points through a tube containing pure solvent only (blank), the galvanometer reading,  $G$ , obtained on insertion of the colored sample solution, will represent the percentage of light transmitted by the colored solution in terms of the uncolored blank. The density  $L$ , is then equal to  $-\log \frac{G}{100}$ , or

$$L = 2 - \log G$$

The  $L$  readings corresponding to given galvanometer deflections as found in the  $L-G$  table accompanying the colorimeter represent the density of the solutions essentially as they would be measured spectrophotometrically.

*Vitamin A*—The  $L$  value unit here used for the expression of vitamin A in plasma is calculated directly comparable to the unit used by Dann and Evelyn for the vitamin A in cod liver oil.<sup>2</sup> This value was in turn made as closely analogous as possible with the familiar spectrophotometric unit  $E$  (1 cm., 1 per cent, 620  $m\mu$ ), and is written  $L$  (1 cm., 1 per cent, 620  $m\mu$ ). Since the effective layer of solution through which the light passes in the Evelyn colorimeter is 1.9 cm. instead of 1 cm. (spectrophotometer), this difference is equalized by introducing the factor  $\frac{1}{1.9} = 0.525$  in the calculation of the  $L$  value. In the formula of Dann and Evelyn,

$$L \text{ value (oil)} = \frac{52.5}{M} \times L_{90} \quad \text{--- (1)}$$



where  $M = \text{mg. of oil in the sample taken for analysis}$ . Then in the final 10 c.c. of solution read in the colorimeter tube there are represented  $M/10 \text{ mg. of the original oil per cubic centimeter of solution, or } (1/1,000 \times M/10) \times 100 = M/100 \text{ gm. per 100 c.c. of solution}$ . Then the  $L$  value (oil) (1 cm., 1 per cent, 620  $m\mu$ )

$$m\mu) = \frac{1}{1.9} \times \frac{1}{M/100} \times L_{620}, \text{ which on solution yields formula (1) above.}$$

In the case of blood, where the concentration of the vitamin is much lower than in oil, the sample for analysis is taken in terms of cubic centimeters, instead of mg. of material—i.e., 1,000 times as large in order to furnish readable colors. In this case, if  $A = \text{c.c. of plasma used in an analysis}$ , then  $A/10 = \text{c.c. of plasma represented per cubic centimeter of final solution in the colorimeter tube, and } 100 \times A/10, \text{ or } 10A = \text{c.c. of plasma represented per 100 c.c. of solution}$ . Then

$$L \text{ value (plasma)} = \frac{1}{1.9} \times \frac{1}{10A} \times L_{620} = 0.0525 \times L_{620} \text{-----}(2)$$

*Correction for Carotene.*—Since the carotinoids in plasma also react with antimony trichloride to produce blue color in excess of that produced by the vitamin A, a correction for the carotinoids must be applied to the final calculation of the vitamin. This is done according to the relationship  $L_{620}/L_{440}$ , given by Daun and Evelyn for pure samples of carotene. Accordingly, the corrected formula for vitamin A in plasma becomes

$$L \text{ value (plasma, corrected)} = \frac{0.0525 [L_{620} - (0.14 L_{440})]}{A} \text{-----}(3)$$

In the routine here adopted for blood analysis,  $A = 10 \text{ c.c. of an original 12 c.c. of petroleum ether extract from 5 c.c. of plasma—i.e., it represents } \frac{5}{6} \times 5 \text{ c.c. of the original plasma}$ . When these quantities are adhered to

$$L \text{ value (plasma, corrected)} = \frac{0.0525 [L_{620} - (0.14 L_{440})]}{25/6} \\ = 12.6 [L_{620} - (0.14 L_{440})] \times 10^{-3} \text{-----}(4)$$

If more or less than 5 c.c. of plasma are used in the original extraction, the appropriate change is made in the factor 12.6.

It is to be noted that the plasma values given in the accompanying tables represent, for convenience in expression, 1,000 times the actual  $L$  values calculated from formula (4), that is, they represent  $L \text{ value} \times 10^{3*}$

*Carotene Concentration.*—If record of the concentration of carotene in the plasma as mg. per 100 c.c. is desired, this may be estimated from the  $G_{440}$  reading by reference to a standardization curve like that of Fig. 1. This is simply an experimental curve obtained by reading known dilutions of a sample of pure carotene in petroleum ether with filter 440 against a petroleum ether blank. The material used as standard in the present work was from a freshly opened ampoule of crystalline carotene prepared by the General Biochemicals, Inc., Division of the S.M.A. Corporation of Cleveland. Because of the difficulty of preparing strictly pure carotene, different samples may yield somewhat different standardization curves.

\*Using the factors of Holmes and Corbet for pure vitamin A (Holmes, H. N., and Corbet, R. E.: J. Am. Chem. Soc. 59: ii, 2042, 1937) and that of Daun and Evelyn for equivalence of the  $L$  value unit, it is possible to transpose these figures for  $L$  value in blood to their approximate equivalents in terms of International Units. In this way it is estimated that a solution with an  $L$  value of 1 would contain something like 58,500 I. U. of vitamin A per 100 c.c.

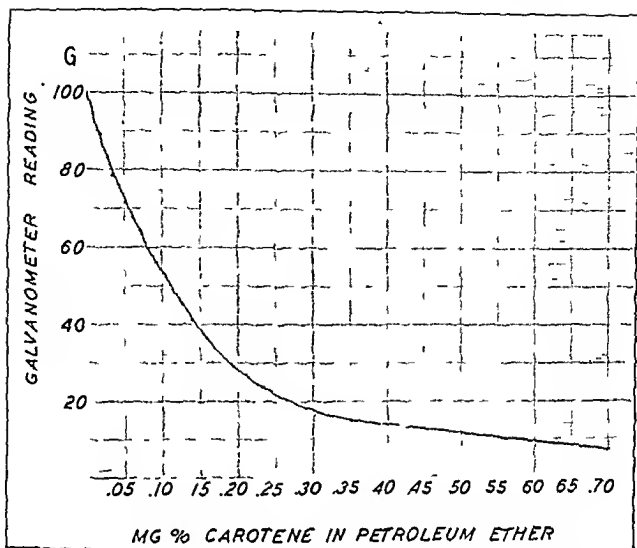


Fig 1—Standardization curve of pure carotene in petroleum ether Percentage transmission at 440 mμ

FACTORS FOR CONVERTING READINGS FROM FIG 1 TO CAROTENE CONCENTRATION IN PLASMA

WHEN $A = 10/12 \times \lambda$ CC OF PLASMA EXTRACTED	MULTIPLY CAROTENE READ FROM THE CURVE BY THE FOLLOWING FACTOR
cc of plasma used	
5.0	4
4.5	67
4.0	0
3.5	43

Dann and Evelyn found that the color of pure carotene in chloroform followed Beer's law, as does the blue color of the antimony trichloride reaction—i.e., that it showed a constant relationship between density ( $L$ ) and amount of material in solution. They report an  $L$  value of 1,440 for their sample of pure carotene. Calculation of the  $L$  values ( $\frac{525}{M} \times L_{440}$ , where  $M$  = amount of the chromogen in each sample solution read) from Fig 1 shows that the color of the carotene here dissolved in petroleum ether, rather than chloroform, has not followed Beer's law, i.e., the  $L$  value is not a constant. The values figure very close to 1,440, however, in the middle range of this curve, i.e., for galvanometer readings between about 24 to 64, which range covers most of the plasma values found.

#### RESULTS IN HUMAN PLASMA

Using the method here given, it has appeared that the substance in human plasma responsible for the Carr-Price reaction (corrected for the previtamin carotinoids), as shown in formula (3), is present in quite constant concentrations

in the plasma of healthy individuals, and that it evidently changes little under constant physiologic conditions, unless vitamin concentrates are being taken.

TABLE I  
FASTING VERSUS POSTPRANDIAL BLOOD

SUBJECT	BLOOD TAKEN	CAROTENE MG. %	A L VALUE $\times 10^3$
1	a. Fasting b. Two hours after meal high in butter, cream, and milk. Heavy lipemia	a. 0.20 b. 0.23	a. 2.38 b. 2.32
2	a. Fasting b. Two hours after meal including bacon, butter, milk, and cream. Moderate lipemia	a. 0.25 b. 0.26	a. 2.69 b. 2.51
3	a. Fasting b. Six hours after breakfast and three hours after lunch including fruit, lettuce, butter, milk. Moderate lipemia	a. 0.19 b. 0.18	a. 1.66 b. 1.60
4	a. Fasting b. Two hours after breakfast with egg, butter, and cream, plus 1 teaspoonful of haliver oil. Moderate lipemia	a. 0.14 b. 0.16	a. 3.50* b. 9.11
5	a. Fasting b. Two and a half hours after breakfast with fruit, egg, butter, and cream, plus 1 teaspoonful of haliver oil. Moderate lipemia	a. 0.15 b. 0.14	a. 2.76* b. 6.90

\*High fasting values in these cases had resulted from previous dosage with haliver oil, the last doses having been taken twenty-six and twenty-seven hours before the present experiments.

*Fasting Versus Postprandial Values for Blood A.*—It was observed (Table I) that, although a very large dose of the vitamin—teaspoonful of haliver oil—more than doubled the A in an already high blood within two hours, ordinary meals did not increase the concentrations of either carotene or A in the plasma within two to six hours, even though lipemia was definite in the postprandial samples. Hence the following studies are not restricted to fasting blood, but include as well samples taken either in midmorning or midafternoon.

*Prompt Versus Delayed Analysis for Blood A.*—Blood was taken with potassium oxalate as an anticoagulant, and the plasma was separated promptly by centrifugation. The determinations to be reported are about equally divided between those done immediately after the blood was withdrawn and those in which the separated plasma was kept in the refrigerator for from a few hours to overnight before analysis. The figures of Table II indicate that there was no significant loss of chromogenic power when the plasma was stored in the cold for a day before extraction.

TABLE II  
DELAYED ANALYSIS

SUBJECT	CONDITIONS OF ANALYSIS	CAROTENE MG. %	A L VALUE $\times 10^3$
1	a. Prompt analysis b. Plasma kept in refrigerator 20 hours	a. 0.09 b. 0.09-	a. 1.75 b. 1.70
2	a. Prompt analysis b. Plasma kept in refrigerator 22 hours	a. 0.25 b. 0.24	a. 2.32 b. 2.35

*Subjects Used*—Table III shows the figures for blood A and carotene obtained in a series of 30 male and 34 female control subjects in good health. The subjects were all connected with the clinical or laboratory staffs of the hospital and on uncontrolled dietaries, most of them felt from their descriptions to be at least adequate, and many more than the average liberal in A containing foods. Correlation studies with dark adaptation measurements available on the same individuals will be included in later reports. The effect of large doses of A concentrates, with and without various supplements, is also being followed in a number of the subjects.

TABLE III  
CAROTENE AND VITAMIN A IN PLASMA OF NORMAL CONTROLS

MALES				FEMALES			
SUBJECT	DATE	CAROTENE MG %	A L VALUE X 10 <sup>3</sup>	SUBJECT	DATE	CAROTENE MG %	A L VALUE X 10 <sup>3</sup>
1	3/22	0.12	2.60	1	9/12	0.17	1.42
	5/ 5	0.17	2.93	2	9/12	0.21	1.32
2	3/ 2	0.12	1.90		9/13	0.19	1.38
3	4/26	0.08	1.48	3	9/12	0.17	1.36
	10/11	0.18	1.79	4	9/13	0.19	1.59
4	4/26	0.05	1.59	5	9/14	0.24	1.58
5	4/29	0.10	1.75	6	9/14	0.17	1.27
6	5/ 4	0.10	2.02	7	9/17	0.14	1.13
7	5/ 4	0.13	2.18		9/21	0.10	1.13
8	5/ 4	0.07	1.82	8	9/17	0.18	1.10
9	0/17	0.15	2.28		9/21	0.14	1.37
	0/12	0.20	2.38	9	9/17	0.11	1.04
	0/12	0.23	2.32	10	9/19	0.29	2.22
10	9/14	0.29	2.40	11	9/20	0.14	1.60
	10/13	0.25	2.69	12	9/21	0.09	1.33
11	9/19	0.14	3.14	1	9/21	0.28	1.77
	9/21	0.17	3.30	14	9/26	0.24	1.33
12	9/19	0.18	1.53	15	9/26	0.09	1.11
13	9/19	0.20	1.76	16	9/26	0.15	1.17
14	9/19	0.14	2.04	17	9/26	0.14	1.33
15	0/20	0.13	2.07	18	9/27	0.21	1.19
16	9/20	0.13	2.05	19	9/27	0.18	2.00
17	9/20	0.14	1.66	20	9/27	0.24	1.32
18	9/20	0.15	2.07	21	9/30	0.32	1.92
19	9/27	0.20	1.89	22	10/ 7	0.24	1.69
20	9/27	0.30	1.76	23	10/ 7	0.17	1.62
21	10/7	0.20	2.26	24	10/ 7	0.16	2.04
22	10/7	0.30	1.55	25	10/11	0.20	1.65
23	10/12	0.14	1.76	26	10/11	0.24	1.80
24	10/12	0.12	2.52	27	10/12	0.22	1.51
25	10/14	0.25	2.32	28	10/12	0.28	1.40
26	10/14	0.21	2.62	29	10/13	0.19	1.66
27	10/17	0.18	2.96	30	10/13	0.14	2.82
28	10/17	0.18	2.20	31	10/13	0.23	1.97
29	10/18	0.10	2.66	32	10/17	0.20	1.25
30	10/25	0.14	2.44	33	10/17	0.25	1.84
				34	10/18	0.13	1.48
Average		0.166	2.18*	Average		0.187	1.56*
Standard deviation		0.059	0.50	Standard deviation		0.077	0.37
Coefficient of variability		0.36	0.229	Coefficient of variability		0.41	0.237

\*See footnote on bottom page 1058

*Plasma Vitamin A in Healthy Controls*—The values in Table III, represented in Fig 2, show an unanticipated but definite sex difference for concentration of the vitamin in plasma, the lower figures predominating in the female

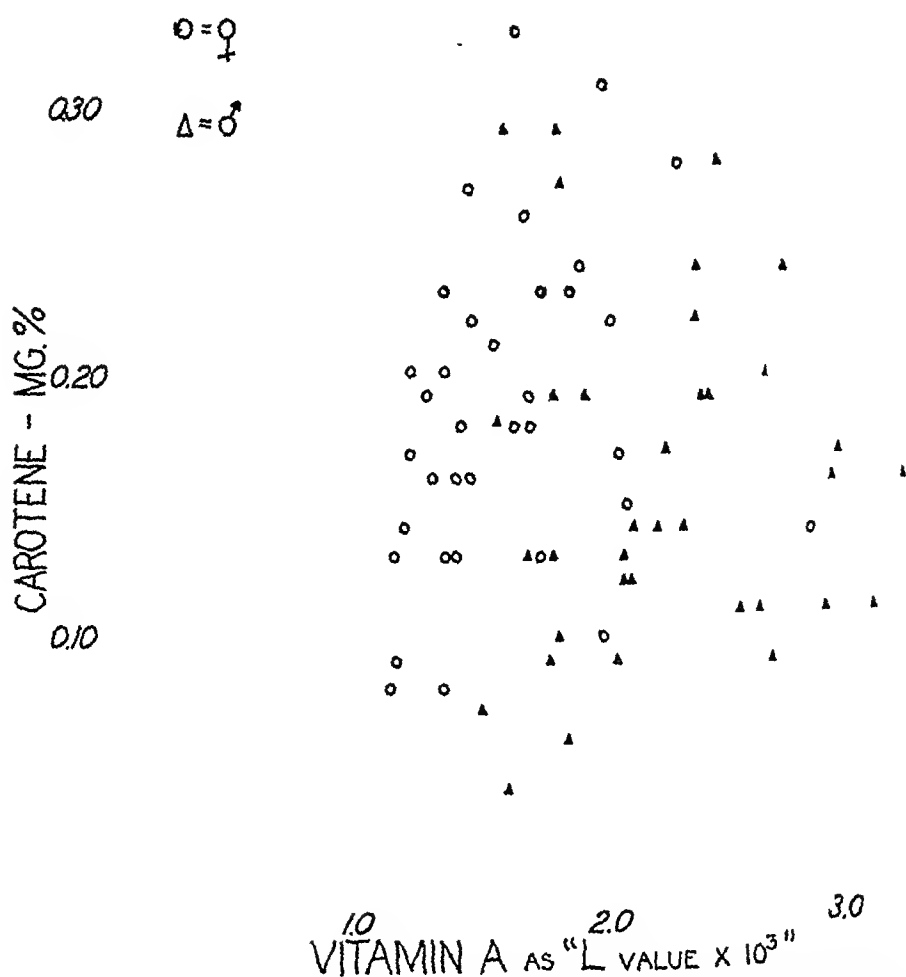


Fig. 2—Lack of correlation between plasma vitamin A and carotene levels in healthy subjects.  $\Delta$  = males,  $\circ$  = females. A sex difference in the concentration of A appears in the distribution of the points.

group. The average value found in 36 determinations on the 30 male subjects was  $2.18 (\times 10^{-3})$  L value units, with a range from 1.48 – 3.30, and a standard deviation of 0.50 (coefficient of variability\* = 0.229). In 37 determinations on the 34 females, the corresponding average was 1.56, the range from 1.11 – 2.82, and standard deviation of 0.37 (coefficient of variability = 0.237). Only 3 of the values for females fell above 2.0, while 22 were above this figure for the males. If this difference were due to relatively larger plasma: cell volumes in the female, carotene, as well, would be expected to vary in the same direction, whereas on the contrary, the carotene for these female subjects ran somewhat higher than for the males. If the physiologic demands for A in the female fluctuate with the hormone cycle—a problem which needs further investigation—one would expect to find greater variability in an unselected series of women than one of men, though this has not been definite in the present comparisons (coefficients of variability, above).

\*Coefficient of Variability = the Standard Deviation expressed in terms of the mean value for the series, thus becoming comparable for series with their means at different levels.

Evidences of a sex difference in the ability to store and retain A in the liver of experimental animals were discovered in the literature after the foregoing observations were made on human plasma. In the protocols of Logaias and Drummond<sup>5</sup> it appears that female rats store more A in the liver under experimental conditions that tend to conserve the stores than do the males. Disappearance of A from the livers of depleted rats was slower in the females or in castrate males than in the male animals studied by Bult and Seigristen.<sup>6</sup>

*Plasma Carotene in Healthy Controls*—There was a slight difference between men and women in the average figures for concentration or variability of plasma carotene in the above series of controls, with the tendency toward higher concentrations and greater variability in the females. The average for the males was 0.166 mg per cent, and for the females 0.187 mg per cent. The values ranged for the males from 0.05 - 0.30 and for the females from 0.09 - 0.34, with coefficients of variability of 0.36 and 0.41, respectively. Fig. 2 shows that there is no consistent correlation between vitamin A and carotene values in the blood.

#### TESTS OF DEPENDABILITY OF THE METHOD

*Reproducibility and Consistency of Figures*—Since pure crystalline vitamin A is not yet available for a known standard, judgments of the dependability of the present method must rest on tests of its consistency and reproducibility. Table IV shows the order of accuracy with which results have been reproduced in duplicate aliquots, and Table V the degree of constancy which has been noted at different times in the same individual under presumably constant physiologic conditions. The one significant change noted was a considerable increase in both carotene and vitamin A in subject 3 male (No. 6, Table V), following a late summer vacation. Experience with the biophotometer has suggested to some observers a seasonal variation with tendency to lower winter and higher summer readings. Examination of larger series will undoubtedly bring out further differences and it is anticipated that more extended studies will reveal the basis for different groupings among the undifferentiated series of Table III.

From Table IV, experiments 5, 6, and 7, it is apparent that a significant part of the experimental error of the method is in the original extraction, rather than in the subsequent steps of the determination. The objectivity of the instrument and the ability it affords to make the readings at the absolute maximum of the color curve remove sources of inconsistency and uncertainty inevitable with the older methods, and clear the way for more comprehensive studies.

*Stability*—The figures of Table II, and experiments 7 to 10 in Table IV, indicate that the vitamin is present in blood in a form that is relatively stable, since neither prompt analysis nor anaerobic handling appears necessary for consistent recovery.

*Chromogenic Powers of Different  $SbCl_3$  Reagents*—Because the accuracy of the Carr Price reaction has often been challenged on the suspicion of variable chromogenic powers of different antimony trichloride reagents, or of the same lot of reagent under varying conditions, the experiments of Table VI were made to test this suspicion under the not at all rigid conditions of control of the present technique. Satisfactory checks were obtained in duplicate analyses made

TABLE IV  
DUPLICATE CHECKS

EXPER. NO.	SOURCE OF MATERIAL	CAROTENE MG. %	A L VALUE $\times 10^3$	REMARKS
1	Pooled plasma	0.17 0.17	1.59 1.60	Duplicates from pooled extract
2	Pooled plasma	0.11 0.11 0.11	1.45 1.42 1.42	Triplicate aliquots from pooled extracts, using two different reagents
3	Pooled plasma	0.21 0.21 0.21	1.77- 1.77- 1.77+	Triplicate aliquots from pooled extracts, using same reagent
4	Pooled plasma	0.13 0.13	1.54 1.52	Duplicates from pooled extract
5	Undiagnosed cachexia	---- ----	0.517 0.471	Duplicate analyses from separate extracts
6	Same patient as No. 5, five days later	---- ----	0.532 0.517	
7	Pooled plasma	(a) 0.15 0.15	(a) 1.51 1.50	
	(a) = aerated with CO <sub>2</sub>	(a) 0.12 0.12	(b) 1.56 1.56	Duplicate analyses from separate extracts
8	Control plasma	(a) 0.06 (b) 0.06	(a) 1.59 (b) 1.60	Duplicates from pooled extract
9	Control plasma (b) = aerated with room air	(a) 0.10 (b) 0.10	(a) 1.48 (b) 1.48	
10	Control plasma	(a) 0.04 (b) 0.04	(a) 1.63 (b) 1.59	

TABLE V  
SAME INDIVIDUAL AT DIFFERENT TIMES

NO.	CASE	SEX	DATES	CAROTENE MG. %	A, L VALUE $\times 10^3$
1	Control	M	3/22/38 5/ 5/38	0.12 0.17	2.60 2.93
2	Control	M	6/17/38 9/12/38	0.15 0.20	2.28 2.38
3	Control	M	9/19/38 9/21/38	0.14 0.17	3.14 3.30
4	Ulcerative colitis	M	6/16/38 6/18/38	0.06 0.07	0.67 0.69
5	Control	M	9/14/38 10/13/38	0.29 0.25	2.40 2.69
6	Control	M	4/26/38 10/11/38	0.08 0.18	1.48 1.79
7	Control	F	9/12/38 9/13/38	0.21 0.19	1.32 1.38
8	Control	F	9/17/38 9/21/38	0.18 0.14	1.19 1.37
9	Control	F	9/17/38 9/21/38	0.14 0.10	1.13 1.13
10	Diabetic	F	5/13/38 5/16/38	0.096 0.096	2.29 2.38
11	Cachexia	F	4/ 1/38 4/ 6/38	---- ----	0.494 0.524

TABLE VI  
CHECKS ON ANTIMONY TRICHLORIDE REAGENT

EXPER NO	SOURCE OF MATERIAL	SbCl <sub>3</sub> REAGENT USED	A VALUE × 10 <sup>3</sup>	SOURCE OF THE SbCl <sub>3</sub>
1	Control blood	a Solution 5 days old	2.28	Merck's reagent, mfg date unknown
		b Solution 4 weeks old (In refrig. between use)	2.34	Merck's reagent, mfg date unknown
2	Diabetic blood	a Solution one day old	1.17	Merck's reagent, mfg date unknown
		b Solution 2 months old (Kept at room temp.)	1.11	Merck's reagent, mfg date unknown
3	Pooled extract	Solutions made from different known stocks of SbCl <sub>3</sub> (Kept at room temp.)	1.45	Stock No 1 Merck's reagent, date 32587
			1.42	Stock No 2 Merck's reagent, date 33427
			1.42	
4	Pooled extract	Same conditions as in Experiment 3	1.54	Stock No 1, as above
			1.51	Stock No 2 as above
			1.54	Stock No 3 Mallinckrodt's analyst 3624, control G N H 1
			1.52	

with old and new solutions from the same reagent stock, and with antimony trichloride on hand from different stocks

#### SUMMARY

The Dann and Evelyn photoelectric determination of vitamin A in oils by the Carr Price reaction has been adapted for the assay of 35 to 50 cc of plasma. The technique differs from a prior microadaptation by C. D. May in using the Evelyn macro- instead of the microcolorimeter. With the use of this instrument a number of material simplifications in procedure have been found safe, with recoveries reproducibly consistent under a variety of conditions.

Results for plasma carotene and vitamin A are given for 30 men and 34 women in good health. While the figures for carotene were slightly higher on the average in the female group, those for vitamin A were found distinctly higher for the males than for the females, with averages  $2.18$  and  $1.56 \times 10^3$  "L value" units roughly equivalent to 127 and 91 IU of vitamin A per 100 cc of plasma, respectively. Coefficients of variability for the vitamin values in the two groups were 0.229 and 0.237.

Vitamin A appears to be present in the plasma in a relatively stable form, and to vary little under constant physiologic conditions in individuals who are not taking oil concentrates. More extended studies will doubtless reveal different classifications among general groups of a healthy population.

#### REFERENCES

1. Evelyn, K. A. A Stabilized Photoelectric Colorimeter With Light Filters, *J. Biol. Chem.* 115, 63, 1936.
2. Dann, W. J., and Evelyn, K. A. The Determination of Vitamin A With the Photoelectric Colorimeter, *Biochem. J.* 32, 1003, 1938.
3. May, C. D. Program, American Institute of Nutrition Meeting, Baltimore, 1938.
4. Evelyn, K. A., and Gibson, J. G., II. A New Type of Absorption Cell for the Photoelectric Micro Colorimeter, *J. Biol. Chem.* 122, 391, 1938.
5. Logarås, G., and Drummond, J. C. Vitamin A and the Thyroid, *Biochem. J.* 32, 964, 1938.
6. Bult, A. R., and Sorgdrager, C. J. The Influence of Sex and of Castration of the Males on the Vitamin A Metabolism of the Rat, *Acta brev. Neerland.* 8, 114, 1938.



## EFFECTS OF ANESTHETIC DRUGS UPON RATS TREATED WITH SULFANILAMIDE\*

JOHN ADRIANI, M.D., NEW YORK, N. Y.

**S**ULFANILAMIDE therapy has invaded practically every field of medicine. Surgical patients frequently receive the drug before operation. The anesthetist is often confronted with a patient who has recently had or is undergoing intense sulfanilamide therapy. Recent reports of toxic effects from the drug and particularly the untoward reactions when given with magnesium sulfate or other depressing drugs,<sup>1</sup> have focused attention upon the possibility of dangerous sequelae when narcotics or anesthetics are to be administered during the time when sulfanilamide is present in the body.

The pigmentation or skin color changes that follow administration of sulfanilamide, at first thought due to alteration in the oxygen-carrying pigment, and later observations revealing hemolytic anemia or granulocytopenia, have directed attention of anesthetists to possible asphyxial reactions.<sup>2</sup> Circulatory effects in man during anesthesia have suggested this in a few observations not yet confirmed. More recently, Marshall<sup>3</sup> has shown that toxic doses depress the cortex of the dog, producing results which suggest a decorticated animal. Many hypnotic and anesthetic drugs act upon the cortex. Therefore, attention is also directed toward unfavorable results that may be associated with cortical depression.

The present study was undertaken to observe the reactions of animals treated with sulfanilamide and subjected to anesthesia with the various drugs in current use. Rats were selected as the experimental animal. The drug is detoxified similarly in rats and human beings, by conjugation with acetic acid.<sup>3</sup> The toxicity in rats is low and the absorption slower than in human beings when the drug is given orally or subcutaneously.<sup>4</sup> The animals used in this study were a special strain of white rats, 6 months old, weighing approximately 200 gm. each.

The administration of sulfanilamide to a number of animals is simplified by preparing solutions of the drug. Such solutions were prepared with normal saline as a diluent. Injection was made intraperitoneally with solutions at constant temperature of 37° C. Doses employed amounted to 0.5-1.0 mg. per gram of rat daily.<sup>5</sup> The animals were treated for three consecutive days before anesthesia was administered. Determinations of sulfanilamide in the blood were not undertaken.

The following observations were completed using treated rats (1.0 mg. per gram) with a similar number of untreated controls.

\*From the Division of Surgery, Department of Anesthesia and Laboratory of Experimental Surgery, New York University College of Medicine, New York.  
Received for publication, January 11, 1939.

1 *Reaction to Asphyxia*—Twenty rats were used with 20 untreated controls. On the third day of treatment, three hours after sulfanilamide had last been given, the rats were placed under a large bell jar and nitrogen added continuously but slowly. With the lowered oxygen tension dyspnea was first observed without marked cyanosis. The degree, time of onset, and resultant effects were similar in the treated and untreated groups. Symptoms of asphyxia were marked in all rats after eight minutes' administration of nitrogen. After five minutes' further observations nitrogen was again added until all the animals showed syncope. The concentration of nitrogen was not determined. Oxygen was then added to replace the nitrogen. Recovery of all rats occurred in three minutes. Recovery was similar in the treated and untreated rats and there was no apparent difference between the groups during the subsequent three hours when they were closely observed. No difference was noted in their behavior during the next several days.

2 *Reaction to Excess Carbon Dioxide*—Twenty treated and the same number of untreated rats of the same size and age were placed in a bell jar. Carbon dioxide was gradually added to slowly displace the air. All the rats showed a well defined hyperpnea in less than one minute. The flow of carbon dioxide was continued ten minutes, or until respirations were depressed and the rats became unconscious. Rate and depth of respirations and the onset of unconsciousness were similar for treated and untreated rats. Anesthesia with carbon dioxide was maintained for one to two minutes. Respirations of the treated animals appeared to be predominantly abdominal while in the untreated rats they were more of the intercostal type. After the bell jar was removed, recovery was no different in either group. No delayed reactions were noted.

3 *Reactions to Nitrous Oxide Narcosis*—Ten treated and 10 untreated rats were placed in a bell jar, and nitrous oxide was gradually admitted. After eight and one half minutes all animals were unconscious. Every animal exhibited marked cyanosis and a well developed hyperpnea. Anesthesia was maintained for four minutes. Recovery was permitted by quickly introducing oxygen. The onset of unconsciousness, hyperpnea, cyanosis, and the signs and symptoms of narcosis were identical in all animals. Results were very similar to those observed in the experiment where nitrogen was used to produce asphyxia. None of the treated or untreated animals died.

4 *Reactions to Ether Anesthesia*—Twenty treated rats were placed in a bell jar with 20 controls, and oxygen saturated with ether vapor was slowly added. After five minutes all rats were unconscious. They were maintained in this narcotic state for five minutes, during which time treated and untreated rats responded similarly. Ether was then added until (after four to six minutes) respiratory arrest occurred. Reactions of treated rats and the controls were similar, and there was no difference in the recovery. An identical number of the treated and the controls required artificial resuscitation. All rats recovered completely.

5 *Reactions to Cyclopropane Anesthesia*—Eighteen treated rats were placed in a bell jar with 18 controls, and cyclopropane was slowly added. Within three and one half minutes all the rats were anesthetized. The onset

TABLE I

DRUG	DOSE MG./KG.	TREATED				UNTREATED			
		ANIMALS	HYPNOSIS ONSET	HYPNOSIS DURATION	ANESTHESIA ONSET	ANESTHESIA DURATION	MOR- TALITY	ANIMALS	HYPNOSIS ONSET
Group 1 Thio-ethylmethyl	60	18	5.0	*	3.3	2 hr. +	30%	18	5.6
Group 2 Thio-ethylmethyl	60	4	4.0	1-2	--	--	0	4	3.2
Group 3 Evipal	80	10	2.7	20.7	5.5	14.3 min.	0	10	3.1
Pentothal	50	10	2.4	*	1.9	3 hr. +	50%	10	3.3
Pentothal	40	8	2.0	*	2.0	3 hr. +	60%	8	3
Amytal	70	9	6	†	9	4 hr.	66%	9	6
Nembutal	40	16	2.1	*	4.0	5 hr. +	50%	16	4.4
Nembutal	20	10	5	1½-2	10	30 min.	0	10	6

\* Undetermined—extended many hours.

Group 1.—Results following administration of thio-ethylmethyl 60 mg. per kg. body weight to a group of rats, one-half of which had been treated with 0.5 mg. sulfanilamide per gram body weight for three consecutive days.

Group 2.—Rats from the treated group in Table I that recovered from anesthesia and were allowed to rest for four days before reanesthetizing with thio-ethylmethyl.

Group 3.—Results from various barbiturates administered to rats treated with 0.5 mg. sulfanilamide per gram body weight and untreated controls.

(1) Controls not anesthetized.

(2) 50% controls not anesthetized.

(3) Controls not anesthetized.

(4) 66% controls not anesthetized.

of unconsciousness, the respirations and signs of anesthesia were similar in both groups. Anesthesia was maintained for five minutes. When no difference in behavior could be detected, cyclopropane was again added. Five minutes later one half of the rats had respiratory arrest. The number was equally distributed among the treated and control animals. Air was added to effect recovery. Three of the controls did not recover. One treated rat died.

6 *Reactions to Chloroform Anesthesia*—Fifteen rats were placed in the bell jar with 15 controls, and chloroform vapor was slowly added. Anesthesia, evident in three minutes, was maintained for eight minutes with no variation in effects and no difference in respiration for either group. Chloroform vapor was again added until half the total number of animals were at respiratory arrest. Two controls and one treated rat did not recover.

7 *Reactions With Tribromethanol Narcosis*—A 3 per cent solution of tribromethanol in saline (at 37° C) was prepared. One hundred fifty milligrams per kilogram were given intraperitoneally. The interval between injection and the loss of consciousness was noted as the interval of hypnosis. Anesthesia was determined by pinching the tail with a clamp and noting when pain reflexes were abolished. Nineteen treated rats and 19 controls were tested. The interval of hypnosis averaged 19 minutes in the treated and 21 minutes in the controls. Onset of anesthesia was 21 minutes later in the treated and 20 minutes later in the controls. Anesthesia continued for an average of 17.6 minutes in the treated animals as compared to 15.2 minutes in the controls. Duration of the total period of unconsciousness differed but little for the two groups.

8 *Reactions to Injections of Derivatives of Barbituric Acid*—Various barbiturates of the ultra short acting and short acting types that are frequently used clinically were administered intraperitoneally in a 2 per cent solution. The degree of hypnosis was determined by noting the reactions when the tail was pinched. Methyl butyl ethyl thio barbituric acid (pentothal), isoamyl ethyl thio barbiturate (thio ethamyl), and N methyl cyclo hexenyl methyl barbituric acid (evipal) were used in the form of their sodium salts. Isoamyl ethyl barbituric acid (amytal), the oxygen homologue of thio ethamyl and pentobarbital, methyl butyl ethyl barbituric acid (nembutal), the oxygen homologue of pentothal, were also employed. The amount of the drugs given, onset of hypnosis, and onset of anesthesia are tabulated in Table I. Duration of anesthesia which differed in the treated and untreated rats very markedly in some instances, is also tabulated or explained in the text. These rats were given 0.5 mg of sulfanilamide daily instead of 1.0 mg.

Eighteen treated and as many untreated rats were given 60 mg per kg (50 per cent MLD),<sup>7</sup> sodium thio ethamyl intraperitoneally. Hypnosis was evident in the treated rats in 4.2 minutes average, and in six minutes in the untreated group (Table I). Anesthesia, which continued more than three hours, was produced in all of the treated rats. None of the controls became anesthetized. Five of the treated group died without recovering from the anesthesia. Four surviving rats, shown in Table II, were allowed to recover.

for four days. They were then given the same amount of sodium thio-ethyl that had been administered to them previously. Controls were also injected. These four animals now reacted similarly to the controls, quite unlike their reaction when anesthetized during their treatment with sulfanilamide.

Pentothal, another thiobarbiturate, was given in doses of 50 and 40 mg. per kg., with a mortality of more than 60 per cent and 50 per cent, respectively, in treated rats, and 10 per cent and none in the untreated (Group 3).

Evipal, an ultra short-acting barbiturate, not a thio derivative, was administered, 80 mg. per kg. (40 per cent of M.L.D.).<sup>8</sup> All of the animals so treated recovered. The treated rats were anesthetized for an average of fourteen minutes, while for the controls only hypnosis was effected. Treated rats given amytal and nembutal likewise showed a marked difference to controls untreated with sulfanilamide (Group 3).

The animals that died following anesthesia with the barbiturates always developed a Cheyne-Stokes' type respiration and gray cyanosis. Autopsies revealed no significant gross findings. The blood was always very dark and did not coagulate.

Another group of 9 rats was given intraperitoneally sodium thio-ethyl, 60 mg. per kg. of body weight, together with 0.5 mg. of sulfanilamide per gram of body weight. A similar number of controls received the same dose of the barbiturate only. Onset of hypnosis averaged four and one-half minutes in the control group, and none were anesthetized. In those treated with sulfanilamide, hypnosis developed at the same time, but in these animals the hypnosis deepened and lasted for an average of four and one-half hours. Controls recovered completely in this period.

Nine rats were given 60 mg. sodium thio-ethyl per kilogram body weight four hours after receiving one injection of 0.5 mg. per gram of sulfanilamide. Anesthesia was not produced, but hypnosis was greatly prolonged over that of controls. Twenty-four hours after sulfanilamide treatment, the reaction to sodium thio-ethyl anesthesia was similar to that of untreated rats. With 1.0 mg. per kg. per day the reaction was similar, the anesthesia prolonged and the mortality was as high as 60 per cent when 60 mg. thio-ethyl was administered to rats so treated. The reaction was not obtained when less than 0.25 mg. per gram was used daily.

From these data it is obvious that rats treated with sulfanilamide react with little difference to volatile or gaseous anesthetics than do untreated controls. However, rats so treated react quite differently to injected anesthetic drugs than do untreated controls. The barbituric acid derivatives of the short and ultra short-acting groups, which are ordinarily used for surgical anesthesia, are not borne well by rats having had sulfanilamide. These barbiturates are thought to be detoxified in the liver. However, tribromethanol, which is conjugated with glycuronic acid, also in the liver, produces reactions very similar in treated and untreated groups. Delayed detoxification may not be the solution for the unusual narcotic effect of barbiturates. Barbiturates act upon the cortex and hypothalamus to depress the structures there.<sup>9</sup> Toxic doses of sulfanilamide in the dog likewise depress the cortex.<sup>2</sup> The combina-

tion of the two may greatly enhance the depression. Although the wide species variation to barbiturate reactions is established it is suggested that the same combination may not be advisable in human therapy.

#### SUMMARY

White rats were treated with sulfanilamide 0.5 to 1.0 gm per gram body weight daily for three days. These animals and a similar number of untreated controls were given various volatile gaseous and nonvolatile anesthetic drugs. They were also subjected to asphyxia and carbon dioxide excess.

Reactions to the volatile agents ether and chloroform to the gases nitrous oxide and cyclopropane, and to carbon dioxide excess or oxygen want were the same in treated and untreated rats. There was practically no difference in the reaction to tribromethanol (aveitin) in the two groups.

Rats treated with sulfanilamide and then given evipal, pentothal, thio ethamyl, amytal, or nembutal showed effects unlike untreated rats. The sub anesthetic doses became anesthetic and often lethal. Anesthetic doses were usually lethal. The thio derivatives of lumbinic acid were the worst offenders. Rats treated with sulfanilamide and allowed four days for recovery did not show this reaction. It was also less intense early in sulfanilamide therapy and more intense with increased dosage of the drug.

It is suggested that the combination of sulfanilamide and barbiturates may be unwise in human therapy.

Acknowledgment is made to Dr. E. A. Rovenstine and Dr. Charles L. Burstein, Professor and Instructor of Anesthesia, respectively, New York University College of Medicine for assistance in this study and preparation of this report.

#### REFERENCES

- 1 Editorial. A Complication Following Treatment With Sulfanilamide, *J. A. M. A.* 108 1345, 1937.
- 2 Wood, W. B. Anemia During Sulfanilamide Therapy, *J. A. M. A.* 111 1916, 1938.
- 3 Marshall, E. K., Jr., Cutting, W. C., and Emerson, K. Jr. Toxicity of Sulfanilamide, *J. A. M. A.* 110 257, 1938.
- 4 Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C. Toxicity of Sulfanilamide, *Proc. Soc. Exper. Biol. & Med.* 36 245, 1937.
- 5 Report of the Council on Pharmacy and Chemistry. Sulfanilamide and Related Compounds, *J. A. M. A.* 108 1888, 1937.
- 6 Grulwitz, O. M., Dox, A. W., Rowe, L. W., and Dodd, M. C. A Pharmacologic Study of Certain Thio barbiturates, *J. Pharmacol. & Exper. Therap.* 60 125, 1937.
- 7 Burstein, C. L., and Rovenstine, E. A. The Anesthetic Efficiency of Sodium Isoamyl Ethyl Thio barbiturate, *Anesth. & Analg.* 17 201, 1938.
- 8 Levy, J. Pharmacology and Biochemistry of the Barbiturates, *Anesth. & Analg.* 2 177, 1936.
- 9 Nielsen, C., Higgins, J. A., and Spruth, H. C. Pharmacology of the Barbiturates, *J. Pharmacol. & Exper. Therap.* 26 54, 1925.

# LABORATORY METHODS

---

## THE USE OF A REDUCING FACTOR OF PREGNANCY URINE IN THE DIAGNOSIS OF PREGNANCY\*

---

DONALD E. BOWMAN, PH.D.  
CLEVELAND, OHIO

---

IT IS generally agreed that biological assay affords an accurate means of diagnosing pregnancy. The most accurate and commonly employed biologic methods have the disadvantage of involving considerable time and expense. The length of time is especially undesirable in many cases. For these reasons, a purely chemical test as accurate as the biologic methods, but relatively simple in technique and economical in time and materials, should be of distinct value, since it would permit prompt diagnosis and at the same time obviate the need for the special facilities required by the biologic methods. In addition, a chemical test might be expected to yield results somewhat more quantitative in character than the biologic tests now employed and, therefore, aid in interpreting various abnormal conditions.

It was recognized at the inception of this study that preparations of urinary pregnancy hormones contained a reducing factor, and accordingly an attempt was made to utilize this in the development of a chemical test for pregnancy. At first a delicate qualitative reducing reaction was employed,<sup>2</sup> while later use was made of oxidation-reduction dyes as the indicator.<sup>3, 4</sup> The first test described by Visscher and Bowman<sup>2</sup> was found, under properly controlled conditions and in agreement with subsequent experiments of others, to yield an accuracy of 90 to 93 per cent, although with less care, and in the presence of certain interfering substances, the accuracy could be seriously reduced. In an effort to make the test more quantitative in character various oxidation-reduction dyes were next tried.<sup>3, 4</sup> Likewise attempts were made to separate the specific reducing factor from other reducing materials or interfering substances. To accomplish this, most of the reagents which have been reported in the literature as being capable of precipitating the urinary gonadotropic hormones were tried. In testing out the practical value of these several variations of the test, observations were made on urine specimens from well over a thousand cases. Although the test, improved by these modifications, fell short of the desired end in not being sufficiently quantitative or specific in character, it did serve to definitely correlate a reducing factor with the gonadotropic hormone.

\*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

Aided by grant No. 379 from the Committee on Scientific Research of the American Medical Association.

Reported at the Meetings of the Sixteenth International Physiological Congress,<sup>1</sup> Zurich, Switzerland, August 17, 1938.

Received for publication, August 17, 1938.

It was found possible to put the observed reducing action on a definite quantitative basis by resorting to iodimetric titration, although it was obvious that the values obtained were the sum of the specific and other reducing substances in a given preparation. Therefore, electrometric studies were undertaken in order to observe the relative concentrations of the various reducing factors present\*. It was hoped that by following the electrometric titration curves of various preparations in this manner, the most efficient means of purifying the desired reducing factor would be more readily apparent.

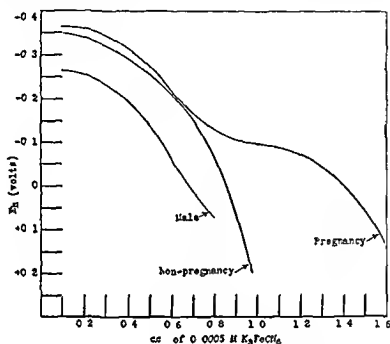


Fig 1—Typical oxidation reduction titration curves

Fig 1 presents typical curves obtained during the course of the study which indicate the presence of the pregnancy reducing factor, as well as an interfering substance unrelated to pregnancy. Following various urinary preparations in this way, it was found that the interfering substances responsible for the upper portion of these curves could be eliminated by fractional precipitation with acetone held at a definite pH with a phosphate buffer solution. This method of separation was then utilized as the basis of a more simple titrimetric technique which has now been employed in a study of over 300 normal cases.

**Method**—The details of the method now employed are as follows. A fresh specimen of urine, having a specific gravity of 1.020 or higher, is brought to a specific gravity of 1.020 with distilled water. Twelve cubic centimeters of the urine are then adjusted to a pH of 7.4 by adding 10 per cent sodium hydroxide, using bromthymol blue on a spot plate as the indicator. Nine cubic centimeters of acetone are added, the tube stoppered, and the precipitate centrifuged down. The supernatant fluid is then poured off and adjusted to a pH about 5.9, with 66 per cent sulfosalicylic acid, using the same indicator. If a precipitate forms at this point, it is centrifuged down and discarded. One cubic centimeter of 1 M Sorensen's phosphate buffer, pH 5.90, is added to the fluid, followed by 18 cc of acetone. This is stirred several minutes, stoppered, and centrifuged for ten minutes. The supernatant fluid is drawn off by suction, carefully leaving the

\*The author wishes to thank Dr. Henry Borsook of the California Institute of Technology for aid in the construction and use of the electrometric titration apparatus.



precipitate and the viscous, well-separated layer of fluid in the bottom of the centrifuge tube. The viscous fluid contains the precipitated phosphate salts in addition to the pregnancy reducing factor and a gonadotropic factor. The precipitate and the viscous fluid are dissolved in 5 c.c. of water. One cubic centimeter of this solution is pipetted into a test tube, avoiding an excess of any insoluble precipitate which may be present. (The use of 1 c.c. also permits a duplicate titration when this is desirable.) After adding a few drops of 5 per cent starch solution, this is titrated with 0.0005 N iodine in a water bath, maintained carefully at 38° C. The oxidation-reduction equilibrium is reached quite slowly, so that in this series the iodine was added in 1 c.c. quantities allowing five minutes between each further addition and continuing until a definite starch-iodine color remained at the end of the five-minute interval. Employing this technique, it has been found to be important that the original specimen had a specific gravity of 1.020 or higher, otherwise some false negative results might be obtained. Effort is being made to reduce this requirement.

TABLE I  
RANGE OF TITRATION VALUES, TERMS 0.0005 N IODINE

	MAXIMUM TITER	MINIMUM TITER	AVERAGE TITER
	c.c.	c.c.	c.c.
Pregnancy	50	10	17.2
Nonpregnancy	5	1	2.33
Male	5	1	2.69

Table I gives the range of titration values obtained in this series when 0.0005 N iodine was used as the oxidant. It will be observed that there was a marked difference between the average titration values for the pregnancy and the nonpregnancy preparations. It may be further pointed out that, with the exception of 3 cases, the pregnancy and the nonpregnancy values had no tendency to overlap. In other words, the nonpregnancy titers did not exceed 5 c.c., while the pregnancy titers exceeded 10 c.c.

TABLE II  
RESULTS OBTAINED WITH PRESENT METHOD

Correct positives	108
Correct negatives (female)	80
Correct negatives (male)	115
False positives	1
False negatives	0
Doubtful results	3
Total number of cases	303
Per cent of accuracy	98.4

Table II presents the data from this same series, arranged in a qualitative manner showing the distribution of cases.

#### DISCUSSION

The identity of this reducing substance is of considerable interest. This is especially true in view of its apparent association with the gonadotropic factor of pregnancy urine. Since the concentration of urinary gonadotropic hormones is the only well-established basis of a biologic test for pregnancy, a chemical test

for such diagnosis would be of more immediate value if it indicated the relative concentration of these hormones

Therefore, it is of particular interest to note that a preparation of the gonadotropic factor of pregnancy urine obtained by repeated reprecipitation with the well known acetone method has many properties in common with the viscous fluid obtained in the chemical pregnancy test here described. Both give positive Mohsch reactions and have gonadotropic activity when injected in young female rats. In addition, the gonadotropic preparation exhibits oxidation reduction properties similar to those shown by the reducing factor employed in the pregnancy test.

Since this paper was completed, Guinn Buchanan and Wilson have made an important contribution to our knowledge of the chemical nature of the gonadotropic hormone of pregnancy urine. In a preliminary note<sup>6</sup> they presented evidence which indicated that the reducing substance contained a galactose component. In their first final publication<sup>7</sup> they describe a method of extracting and purifying the gonadotropic hormone and state that highly purified preparations contain a carbohydrate polypeptide complex, which has the properties of a mucoid. It is of further interest to note that this product slowly reduces ammoniacal silver nitrate in the cold.

The association of the reducing factor and the gonadotropic hormones has been observed in various other ways. In addition to the series of cases reported one patient was followed for a period of three months just prior to pregnancy and again for a similar period beginning with the second month of pregnancy. The urine was examined three times each week, and the rate of excretion of the reducing substance was found to be quite constant in each period. The iodine titer during the period of nonpregnancy varied between 2 and 3 cc, while during pregnancy it varied between 40 and 50 cc.

Similar associations of increased quantities of the gonadotropic hormone and the reducing factor are observed in the urine of adult males with testicular tumor and also in the urine of young boys. Furthermore the concentration of these two urinary factors appears to decrease at about the same time following delivery. Finally, it might be noted that placental tissue extracts containing the gonadotropic factor have been found to contain relatively large amounts of the reducing factor.

It would appear, therefore, that the employment of this pregnancy reducing factor should furnish a valid basis for a chemical test for pregnancy, provided interfering factors have been eliminated. It is particularly significant and important that positive results are obtained with this chemical test about two weeks before the biological tests become positive.

#### SUMMARY

A method of separating a reducing substance found in pregnancy urine from other urinary reducing materials is described. The titration of this reducing substance has been made the basis of a chemical test for pregnancy.

It is believed that the use of this reducing factor provides a valid basis for a chemical test for pregnancy since it occurs in increased quantities in pregnancy urine, and, furthermore, possesses many similarities with a gonadotropic factor.

## REFERENCES

1. Bowman, D. E., and Myers, V. C.: Employment of a Reducing Substance in Pregnancy Urine for the Determination of Pregnancy, *Kongressbericht II des XVI Intern. Physiol. Kong., Zürich, Schweiz.* 173, 1938.
2. Visscher, J. P., and Bowman, D. E.: Chemical Determination of Pregnancy, *Proc. Soc. Exper. Biol. & Med.* 31: 460, 1934.
3. Bowman, D. E., Visscher, J. P., and Mull, J. W.: Possible Application of Chemical Reactions in the Determination of Pregnancy, *Proc. Soc. Exper. Biol. & Med.* 32: 522, 1934.
4. Bowman, D. E., Visscher, J. P., and Mull, J. W.: Properties of Hormones in Female Urine, *J. Biol. Chem.* 109: (proc.) xi, 1935.
5. Gruin, S., Bachman, C., and Wilson, D. W.: The Nature of the Carbohydrate in the Gonadotropic Substance of Pregnancy Urine, *Science* 89: 62, 1939.
6. Gurin, S., Bachman, C., and Wilson, D. W.: The Gonadotropic Hormone of Urine of Pregnancy. I. A Simple Method of Extraction and Purification, *J. Biol. Chem.* 128: 525, 1939.

## NEGATIVE SEROLOGIC REACTION FOR SYPHILIS IN NINE PATIENTS WITH INFECTIOUS MONONUCLEOSIS

JOHN H. MILLS, M.D., AND ELSA JAHN, BALTIMORE, Md.

BERNSTEIN<sup>1</sup> found the Eagle complement fixation test or the Eagle flocculation test for syphilis to be positive in 6 of 37 patients suffering from infectious mononucleosis. Hatz<sup>2</sup> reported a positive Wassermann reaction with negative Kline test in a patient with infectious mononucleosis.

Nine patients at the University Hospital were diagnosed as having infectious mononucleosis in which the clinical diagnosis was confirmed by characteristic hematologic findings and a positive heterophile antibody agglutination titer of  $\frac{1}{256}$  or higher. In 5 of these patients the technique of Bernstein<sup>3</sup> was used. In the remaining 4 patients the rapid method of Straus<sup>4</sup> was used in addition to the Bernstein modification of the Paul-Bunnell test. The Kolmer complement fixation test and a flocculation test (Kline test in two instances, Kahn test in the remainder) were negative in every instance.

While chance may account for the discrepancy between these results and those of Bernstein and Hatz, these observations may be added to the growing literature on the subject.

## REFERENCES

1. Bernstein, Alan: Positive Wassermann in Infectious Mononucleosis, *Am. J. M. Sc.* 196: 79, 1938.
2. Hatz, B.: The Wassermann Reaction in Infectious Mononucleosis, *Am. J. Clin. Path.* 8: 39, 1938.
3. Bernstein, Alan: Antibody Responses in Infectious Mononucleosis, *J. Clin. Investigation* 13: 419, 1934.
4. Straus, R.: Simple Slide and Test Tube Tests for Infectious Mononucleosis, *Am. J. Clin. Path.* 6: 546, 1936.

# STUDIES IN THE PREPARATION OF POLLEN EXTRACTS\*

## II ANALYSIS OF THE SEDIMENT OF POLLEN EXTRACT OBTAINED BY VACUUM FILTRATION ITS QUESTIONABLE SPECIFIC EXCITANT ACTIVITY

H HAROLD GELFAND, M D, GEORGE FLAMM M D AND A J HEFITZ, B S,  
NEW YORK N Y

IN A previous report by Gelfand, Flamm, Center and Heifitz,<sup>1</sup> the authors were able to demonstrate that the occurrence of sedimentation and precipitation of pollen extracts under vacuum filtration is due to the withdrawal of carbon dioxide from the solution by the force of the vacuum. These phenomena were found to be in proportion to the force exerted and to the amount of carbon dioxide thus withdrawn from the buffered saline. The first appearance of a flaky sediment was observed at the measured vacuum pressure of 29.75 inches of mercury. It was further shown that the sediment redissolves upon the addition of carbon dioxide, and that the cloud and sediment reappear when the carbon dioxide is mechanically withdrawn. We suggested the use of carbon dioxide or air pressure filtration rather than suction filtration in order to avoid the possibility of this precipitation and clouding.

In this second report it is our purpose to describe the chemical composition of the sediment formed in pollen extract by the process of vacuum filtration and also to give an account of the activity of the sediment as proved clinically by direct skin tests on pollen sensitive individuals.

### ANALYSIS OF SEDIMENT

Fifty grams of dried and defatted high ragweed pollen were extracted with bicarbonate buffer solution saturated with carbon dioxide, 5 per cent extracts. The solution was divided into two equal parts. One half (extract I) was sterilized by pressure filtration, the other (extract II), by vacuum filtration.

### ANALYSIS OF EXTRACTS

	EXTRACT I	EXTRACT II
Total nitrogen	0.690 mg per cc	0.551 mg per cc
Nonprotein nitrogen	0.340 mg per cc	0.331 mg per cc
Protein nitrogen	0.350 mg per cc	0.220 mg per cc
Protein nitrogen I	0.350 mg per cc	
Protein nitrogen II	0.220 mg per cc	
Difference in protein nitrogen	0.130 mg per cc	

Upon analysis it was found that extract I showed a total nitrogen content of 0.690 mg per cc and protein nitrogen of 0.350 mg per cc. Extract II showed a total nitrogen of 0.551 mg per cc and protein nitrogen of 0.220 mg per cc.

\*From the Department of Allergy, Gouverneur Hospital, New York.

It was, therefore, found that extract I contained an excess of total nitrogen over extract II, amounting to 0.139 mg. per c.c., and that the protein nitrogen\* of extract I was greater by 0.130 mg. per c.c.

The sediment in extract II, resulting from vacuum filtration, was analyzed by the following method: The sediment was collected by decanting the supernatant extract and centrifuging the last portion. The extract was then washed free of inorganic constituents several times with water from which the carbon dioxide had been expelled by boiling, and then dried over phosphorus pentoxide for twenty-four hours in a vacuum desiccator. After this, the sample was weighed and its weight was found to be 0.2 gm. It was then analyzed by microprocedure for nitrogen, carbon, and hydrogen, with the following results:

#### Nitrogen

Weight of sample	6.360 mg.
Net volume of nitrogen	0.145 c.c.
Pressure temperature, 765 mm. Hg	270° C.
Percentage of nitrogen	2.61

#### Carbon and Hydrogen

Weight of sample	8.038 mg.
Weight of water	1.095 mg.
Weight of carbon dioxide	2.135 mg.
Weight of residue	5.528 mg.
Percentage of hydrogen	1.52
Percentage of carbon	7.24
Percentage of residue	68.8

It is evident from these experiments that the sediment obtained by vacuum filtration contained nitrogen, carbon, and hydrogen. The extract which had no sediment had a slightly higher protein nitrogen content.

#### DIRECT SKIN TESTS

After a sediment from ragweed extract in the sample under study was obtained by vacuum filtration, we proceeded to test it for ability to produce cutaneous reactions. Ten patients known to be clinically hypersensitive to ragweed, and who gave positive cutaneous reactions when tested with ragweed saline extract, were selected at random. Scratch tests were made on the inner surface of the arm in the usual manner, the skin being abraded and N/10 sodium hydroxide applied, followed by the application of a small amount of the sediment. A control scratch with N/10 sodium hydroxide alone was also carried out on each patient. All the subjects gave immediate strong reactions, of the positive wheal type, to the sediment (Fig. 1). The reaction of each subject was in direct proportion to the degree of sensitivity of the patient as previously elicited by the direct intracutaneous test with the usual saline extract of ragweed pollen. Thus Class "A" patients, when so treated, showed the largest wheals, and Class "C" the smallest.†

A similar number of patients known not to be ragweed sensitive were tested with the sediment, and their reactions proved to be constantly negative.

\*Protein nitrogen equals phosphotungstate precipitate.

†Class A = most sensitive and Class C = least sensitive as determined by serial dilution tests.

## COMMENT

The powdery sediment formed in ragweed pollen extract, which had been filtered by suction through a Seitz or Berkefeld filter and allowed to stand, has been analyzed quantitatively. The analysis revealed that the sediment contained nitrogen.

This sediment, after being dialyzed and dried, was used in testing patients known to be sensitive to ragweed, as determined by the use of saline extracts. They showed constant and positive reactions by the immediate formation of wheals.

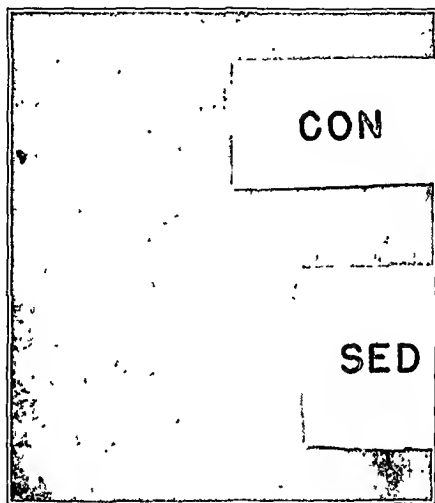


FIG. 1

It is to be seen that in the process of vacuum filtration of ragweed extract, when precipitation and sedimentation occur, it is as a result of alteration in the colloidal solution because of the withdrawal of carbon dioxide by means of suction pressure. This resulted only when the extracting fluid used was the buffered saline recommended by Coca, in which excess carbon dioxide is allowed to bubble through before use.

We have ascertained definitely that alteration in the colloidal solution due to the withdrawal of carbon dioxide will produce the precipitation and sedimentation. Why some batches of pollen will give the sediment and other batches of pollen, although prepared in the same manner, will fail to give it, is unknown to us. One observes that the differences in protein nitrogen in analysis between part I (that sterilized by pressure filtration) and part II (that sterilized by vacuum filtration), give us a figure which for all intensive purposes is rather small and inconsequential in regard to protein nitrogen loss. Hence it is doubtful

whether that little loss of total or protein nitrogen will make any material difference in regard to the activity of the entire solution extract. It is also believed that the positive reaction observed from the dermal test performed by using the sediment as the allergen is probably due to absorption of protein by the sediment rather than the sediment proper.

#### CONCLUSION

In the process of preparation of pollen extracts using buffered saline solution (Coca extracting fluid) the vacuum pressure in the process of suction filtration causes a withdrawal of carbon dioxide with resultant precipitation and formation of sediment. We, therefore, recommend the use of pressure filtration by means of carbon dioxide or air filtration (10 to 20 pounds).

It has been shown that extracts prepared by vacuum filtration have a lower protein nitrogen content than those obtained by pressure filtration, but the amount of loss is so small as to be inconsequential and probably does not alter the activity of the entire solution extract.

The positive reaction obtained when a small amount of the sediment was used as a dermal test on known ragweed sensitive patients is undoubtedly due to the absorbed protein from the solution due to filtration.

#### REFERENCE

1. Gelfand, H. Harold, Flamm, George, Center, J. G., and Heifitz, A. J.: Studies in the Preparation of Pollen Extracts. I. Filtration by Carbon Dioxide Pressure, *J. Allergy* 8: 336, 1937.

---

### TESTS TO SHOW THE POSSIBLE INFLUENCE OF GREASY, ACID, AND ALKALINE TUBES ON THE WIDAL TEST\*

---

WITH SUBSEQUENT REFERENCE TO TUBES SHOWING ACID REACTION

---

F. SMITH, KELOWNA, CANADA

---

TESTS were made to see whether carelessness in preparing glassware might significantly affect the agglutinin-agglutinin reaction.

Many checks were performed which coincided with the results herein quoted and paratyphoid A and B emulsions were checked with positive A and B serum. As the results are to show qualitative possibilities only, we quote just the results with *Br. abortus* and typhoid emulsions and their appropriate specific positive sera. This demonstrates the essential points without sacrificing clearness.

The author is aware that the results are explicable by exact chemical and physical rules, but it is chiefly with the technical aspect that this account is concerned.

1. Tests with serum known to be positive for *Abortus* with standard emulsion of *Br. abortus*:

---

\*From the Okanagan Central Laboratory, Kelowna.  
Received for publication, September 14, 1938.

SERUM DILUTION	1 20	1 40	1 80	1 160	1 320	1 640	1 1280	CONTROL	DESCRIPTION
Br abortus	+	+	+	+	+	-	-	-	Clean tubes, grease less and neutral in reaction
Br abortus	+	+	+	+	-	-	-	-	Tubes rinsed in soap solution and dried in air at room temperature
Br abortus	+	+	+	+	-	-	-	-	Tubes coated inside with a transparent layer of paraffin
Br abortus	+	+	+	+	-	-	-	-	Tubes boiled in soapy water, cleaned with a brush and rinsed 6 times in running water
Br abortus	+	+	+	+	+	+	+	-	Tubes rinsed in normal HCl, dried in room, moisture wiped from rim

Having regard for the work of Michaels, Benish in 1911, and others, we controlled the acid agglutination with two extra tubes of saline and emulsion in acidified tubes as for the test. The controls were negative.

## 2 Tests with known positive typhoid serum and *B. typhosus*

SERUM DILUTION	1 20	1 40	1 80	1 160	1 320	1 640	1 1280	CONTROL	DESCRIPTION
B typhosus	+	+	+	+	+	+	+	-	Tubes properly clean and neutral
B typhosus	+	+	+	+	+	+	-	-	Tubes rinsed in soap solution, dried in air at room temperature
B typhosus	+	+	+	+	+	+	+	-	Tubes rinsed in normal HCl, incompletely dried
B typhosus	-	-	-	-	-	-	-	-	Tubes rinsed with normal NaOH, in completely dried

The same serum and emulsions were used throughout in the case of each of these series.

It seems from these and the other tests we have done in this connection, that agglutination tests may be affected by greasiness, alkalinity, and acidity of tubes, that excess acidity can greatly enhance, and alkalinity can even make negative, a reaction.

With regard to the acidity of tubes and the possibility of using such for increasing a specific reaction, we are doing more detailed tests.



## A SIMPLE INEXPENSIVE PIPETTING MACHINE\*

JOHN H. MILLS, M.D., BALTIMORE, MD.

THOUGH there are inexpensive hand-operated pipetting machines on the market, it was felt that it might be worth while to describe a simple pipetting machine which can be made from a 2 c.c. syringe and a few odds and ends to be found about any laboratory. Such a machine has been in continuous use for the past ten months in the performance of over 5,000 complement fixation tests without the necessity of replacing any of the original parts. Reference to the diagram should make the details of its construction clear.

A 2 c.c. all-glass syringe (2) is mounted in a small wooden block (1) on a suitable baseboard about 8 inches by 12 inches in size. This block is channeled to take the syringe barrel snugly. A groove should be cut in the block near one end of the channel to take the flange of the syringe barrel. Narrow strips of metal (3) go over the syringe barrel and are fastened to the wooden block with screws so that the syringe is held perfectly rigid in the block. There must be no free movement of the syringe barrel whatever. Any similar mounting accomplishing this solidity is satisfactory.

The head of the syringe plunger is fitted into a slot in a metal sling or saddle (4) which is in turn bolted or soldered to a metal lever arm (5). The distance from the point at which the syringe plunger is mounted must be about 8 inches from the fulcrum point (6) so that pressure is not exerted in a sideward direction upon the syringe plunger as the lever is pressed. A slight bit of free play in the mount which holds the syringe plunger head also aids in preventing the plunger from sticking as the lever is pressed.

The lever arm (5) is pivoted at 6 by means of a small stove bolt with washers and lock nuts. Motion must be free. About this point the flat strip making the lever is twisted through a 90° arc at the middle (7).

At 9 is a rigidly mounted set screw which projects through a hole in the lever arm. This hole through which the set screw projects must be sufficiently large so that the set screw does not at any time touch the lever arm. It must, of course, be smaller than the diameter of the head of the syringe plunger. The set screw should be guarded with a lock nut (18). When the lever pulls the syringe plunger outward on its back stroke, the set screw limits the distance of its excursion. Adjusting this screw adjusts the volume delivered at each stroke of the lever. To insure proper filling of the syringe, a suitable number of rubber bands are looped around the set screw block and lever arm so that the syringe plunger is pulled back snugly against the set screw each time the pressure on the lever is released. Experimentation will determine the number of rubber bands necessary for this, as too much tension tends to

\*From the University Hospital, Baltimore.

Received for publication, September 29, 1938.

make the syringe leak air and too little tension prevents accurate complete filling of the syringe to the proper volume.

To reduce friction, the end of the lever arm rides rather loosely upon some smooth thin object, such as a thin glass rod or piece of piano wire (15), which is mounted on the baseboard

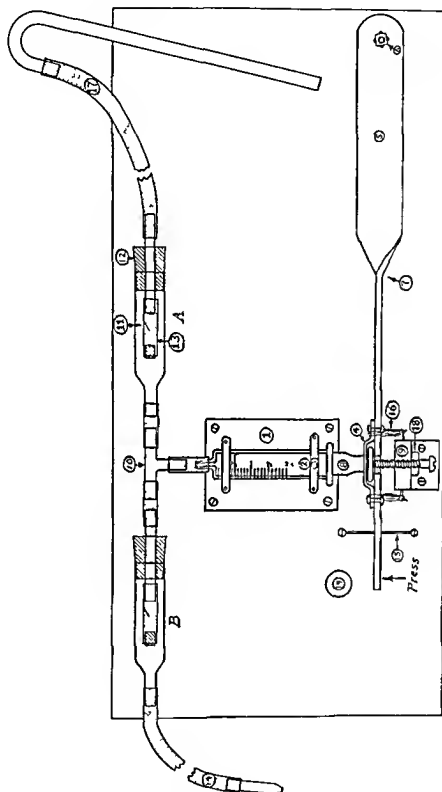


FIG. 1

The tip of the syringe barrel is connected to a glass "T" tube (10). All arms of this "T" tube and all rubber connections should be as short as possible in order to reduce the total volume of fluid contained in the apparatus to a minimum. One arm of the "T" tube connects with the small end of the glass chamber (A). The two valve chambers (A) and (B) are constructed from discarded 10 c.c. transfer pipettes. The end of the bulb is cut off as near the point where it begins to narrow so as to conserve as much of the straight portion of the bulb as possible. A small rubber stopper (12) is fitted into the

large end of this valve chamber. The stopper is pierced by a small bore glass tubing which projects  $\frac{1}{2}$  inch from either end of the stopper. On the end of the glass tube, which projects within the chamber, an ordinary Bunsen valve is fitted (11). A Bunsen valve is merely a piece of gum rubber tubing with a slit approximately  $\frac{1}{4}$  inch long, cut either parallel to the long axis of the tube or at an angle to the long axis (11). A straight smooth slit may be cut with a wet razor blade while the tube is pinched flat, cutting at the sharp bend in the tube. This is the valve commonly used on laboratory wash bottles. Other connections are shown in Fig. 1.

In constructing the valve chambers a few millimeters should be left between the ends and side walls of the Bunsen valves and the chamber walls to allow for slight motion of the valves in opening.

In operation pressure on the lever is exerted with either hand, and the delivery tube is held in the other hand. To insure that all air is removed from the system, the delivery nozzle may be connected with a water vacuum pump to produce a slow flow of fluid through the apparatus. The lever arm is pumped a few times while the fluid flows. This removes the air from the syringe chamber.

Mounting the whole apparatus in a vertical position with the tip of the syringe upward prevents collection of air in the syringe while the apparatus is in use. In such a case, extending the lever arm 8 inches beyond the fulcrum point and using this end as the point at which pressure is exerted during operation will permit of downward instead of the more inconvenient upward pressure. When space is conserved by using glass tubing of internal diameter of not over 4 mm. and rubber tubing of correspondingly small diameter, and all connections as short as possible, the total volume of the apparatus can be kept to less than 30 c.c., thus conserving reagents to be pipetted.

The volume delivered is checked before and after each run by delivering the requisite number of strokes into a small volumetric flask. Readjustment has been found rarely necessary, but is easily made. In calibration, the same speed of stroke, the same amount of tension on the lever, and the same length of tubing in the system should be maintained as is to be used in practice. Washing is easily accomplished by connecting the delivery nozzle with a water pump and filling the reservoir with washing liquid.

The reservoir from which fluid is pipetted should not be placed at too high a level to prevent the fluid pressure created by the siphon to force the valves open. Simple inspection of the apparatus when filled with fluid will readily determine this. The delivery tube (14) should be of thick-walled catheter tubing, about 20 F gauge, to prevent variations in volume delivered due to collapse of the side walls of this tube. A finger rest (19) may be screwed to the baseboard.

A hand-operated instrument offers the advantage of extreme flexibility in rate variation which is not pressed by electrically driven devices and can be operated as fast as the electrical pipetting machines. The advantage of economy is obvious.

## A NEW METHOD FOR CLEANING AND STERILIZING KAHN OR WASSERMANN TUBES

N O GUNDERSON, M D, AND C W ANDERSON, B S ROCKFORD, ILL

THE cleaning and sterilizing of Kahn tubes as it is carried out in various laboratories, is a slow and laborious process. Many laboratories do not have the time or the personnel required to clean every tube with chromic acid cleaning solution before it is used in making the test. Obviously the difficulty in the usual methods lies in the fact that only 10 to 12 tubes can be handled in one operation. This also introduces the personal equation to such a degree that results in some cases may be quite unsatisfactory. As an alternative, this laboratory has been using stainless steel wire cloth baskets 6 inches by 6 inches by 3 inches in size, capacity 140 to 150 tubes for handling the tubes in cleaning, rinsing, and drying operations. The baskets can also be used for cleaning 4 inch, 5 inch, and 6 inch test tubes as well as glass slides.

After the Kahn tests have been completed the tubes are transferred directly from the Kahn racks to the stainless steel baskets. All tubes should be packed uniformly, either with the open end upward or downward, to facilitate rapid rinsing. They are then rinsed several times in a warm alkaline detergent, such as trisodium phosphate, after which they are rinsed in tap water and immersed in chromic acid cleaning solution a minimum of four or five hours and preferably overnight. They are then given at least two rinses in each of three separate portions of fresh tap water, using not less than 5 quarts each time. The pH of the third portion of tap water should be checked occasionally. After this, they are rinsed twice in distilled water and the basket of tubes is placed in a drying oven at 120° C. All tubes which have contained whole blood should be washed and brushed separately in an alkaline detergent before being placed in the baskets for immersion in cleaning solution. Very satisfactory results can also be obtained by first removing the blood clots, then packing tubes in the baskets and immersing in a strong solution of trisodium phosphate allowing them to remain overnight. The following morning rinse several times in tap water, and after giving them a careful inspection, they may be placed in the acid cleaning solution.

Laboratories using over 500 tubes per day might find it advantageous to use larger baskets, 6 inches by 6 inches by 6 inches. In this case one layer of tubes should be packed open end downward and the top layer with open end upward to facilitate rinsing. Baskets, 6 inches by 6 inches by 3 inches, are easily made up from square foot sections of the stainless steel wire cloth. Fig 1 illustrates a simple method of forming the basket.

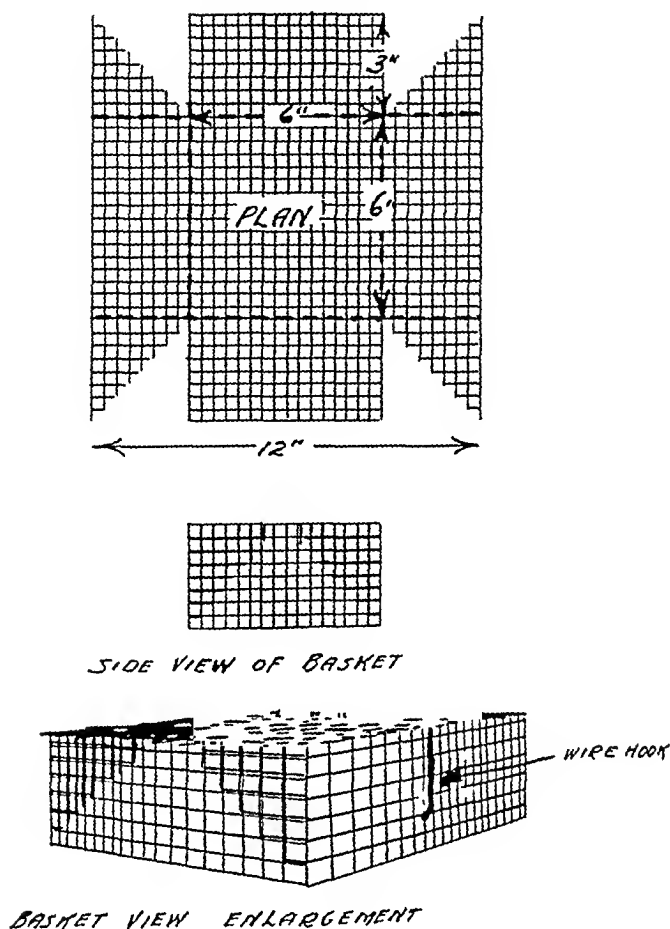


FIG. 1.

Three-inch triangular sections are cut from each corner of the square of cloth, as indicated by the diagram. After removing these pieces, the outer edges of the wire may be turned under by putting the cloth through a timer's erimping machine, thereby removing sharp edges. The basket is then formed by bending the cloth, as indicated by the dotted lines, bringing the remaining triangular section up on the outside of the square end section. This gives added strength to the basket. The loose ends are hooked over into the wire permanently forming the basket. A top is made from a 6 inch square section of the same material and held in place with short sections of stainless steel wire formed into hooks. A more satisfactory basket could be made up by electric welding the end sections rather than hooking them together. Satisfactory tongs can be made up of heavy stainless steel wire for handling the baskets when they are immersed in the acid and during subsequent removal. This laboratory has been using a double pronged  $\frac{1}{8}$  inch lead coated wire hook, 12 inches long, with the upper end twisted to form a handle.

The wire cloth\* used is a 4 mesh stainless steel No. 304, 18 per cent chromium, 8 per cent nickel. Corrosion tests of the wire cloth indicated a loss of ap-

\*Manufactured by W. S. Tyler Co., Cleveland, Ohio.

proximately 0.1 of 1 per cent in one hundred hours at 20° C when the cleaning solution was made according to the formula given in *The Kahn Test*, by Dr R L Kahn, i.e., 252 cc concentrated sulfuric acid, 300 cc water, and 60 gm potassium dichromate. The corrosion rate is increased at higher temperatures and decreased by lower concentrations of sulfuric acid. After about one hundred hours of service, a harmless black coating of nickel and chromous oxide forms on the wire. If used as indicated, the baskets should give four thousand hours or more of service.

These baskets have been used for over one year at the Rockford Health Department Laboratory, and they are still in serviceable condition.

The method here outlined appears to have the following advantages:

- 1 Uniform and complete cleansing inside and diving of each tube
- 2 A marked saving in the cost and time required for cleaning operations
- 3 Breakage of tubes is reduced to negligible quantities
- 4 Reduces to a minimum the danger of acid burns to the individual cleaning tubes
- 5 The cleaning solution lasts much longer

### BLOOD CROSSING BLOCK\*

E E MYERS, M.D., PHILIPPI, W. VA.

A TEST tube support, shown in Fig. 1 has been found very helpful in making the cross matching of blood specimens prior to transfusion.

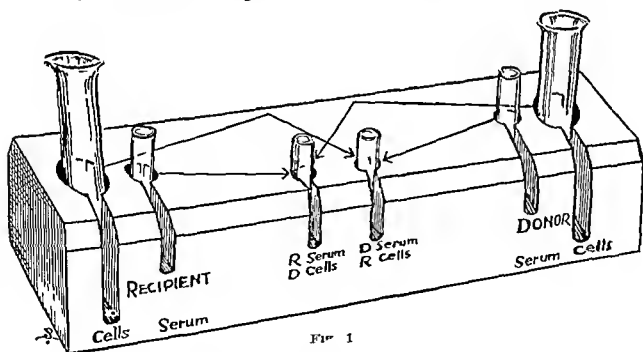


FIG. 1

The block is of wood, painted white, size 10 by 2¼ by 1¾ inches, large holes ¾ by 1½ inches, small holes ⅙ by ⅞ inch. The middle holes have their centers ⅝ inch from the front of the block, and 1¼ inches apart. The end holes are midway between the front and back surfaces of the block, and 1 inch apart, the centers of the large holes being ¾ inch from the ends of the block. The slots are ⅙ inch wide. India ink is used to make the markings.

\*From the Pepper Laboratory, University of Pennsylvania Hospital, Philadelphia.  
Received for publication October 4, 1938.

## A SIMPLE DEVICE FOR DETERMINING APPROXIMATE OXYGEN CONCENTRATION IN OXYGEN TENTS\*

ROBERT W. HUNTINGTON, JR., M.D.,† St. Louis, Mo.

A SIMPLE device used here for determining the approximate oxygen concentration in oxygen tents may merit a brief description, since the omission of such checks in many hospitals is perhaps due in part to a belief that elaborate apparatus is required. As in other bedside analyzers, oxygen is absorbed on moist copper screening with which it comes in contact as the gas sample displaces an ammonia-ammonium salt solution from the absorption chamber. Withdrawal of the sample from the chamber brings the solution back to dissolve the oxide and prepares the copper for the next analysis.

*Design and Construction of the Apparatus.*—The plan of this outfit is best realized from Fig. 1. A calcium chloride U-tube, with 14 cm. sides and provided with side arms and glass stopcocks, serves as absorption chamber and fluid reservoir. The top of the roll of copper gauze in the chamber side is slightly below the bottom of the cock, which is carefully lubricated with stopcock grease or a mixture of this and vaseline. The stopcock of the reservoir side is removed. The sample is measured before and after oxygen absorption in a 10 c.c. Luer syringe, graduated in 0.2 c.c. This syringe must be kept well lubricated with whatever is appropriate for the temperature. Ordinarily vaseline works best, but in very hot weather stopcock grease should be used. A small glass T-tube connects the syringe with the side arm of the chamber and with the small diameter arm of an Ayer three-way stopcock (Beeton, Dickson L/S2), to the opposite arm of which is attached a rubber tube for connection with the tent or other source of gas for analysis. The T-tube should be short and of small bore, and its outside diameter should be about the same as that of the adjoining arm of the metal stopcock and the tip of the syringe, in order that these two joints may each be made with a single piece of rubber tubing. As the outside diameter of the chamber side arm will usually then be larger than that of the T-tube, it may be necessary to cover the T-tube end with fine bore rubber tubing and then use a piece with larger bore to make the connection. The glass and metal ends are brought close together, and shellac is used to make the joints airtight. To reduce the dead space between syringe, three-way stopcock, and chamber, unnecessary length in T-tube or side arm should be filed off before connections are made. Correct analytical procedure minimizes the effect of this dead space. The arrangement is advantageous, since with the glass stopcock shut one may rinse the tent connections by aspirating air from rubber tube *E* into the syringe with the metal cock in position 1, then ejecting the air to the outside with the metal cock in position 2. The ap-

\*From the Department of Pediatrics, Washington University School of Medicine, and the St. Louis Children's Hospital.

†Theron Cattin Fellow, St. Louis Children's Hospital, 1935-38.

Received for publication, October 8, 1938.

piratus is compact enough to be mounted on a small board. The syringe, which need not be of the most expensive grade, and the Ayer stopcock are most easily obtained from a dealer in surgical supplies, while the rest of the equipment is carried by most laboratory supply houses. The total cost of the equipment is about \$6.00.

*Solution in the U-Tube*—In order to dissolve the copper oxide satisfactorily, it appears necessary to use an ammonia ammonium salt solution<sup>1, 2</sup>. With such solutions it is difficult to exclude the possibility of volatilization of ammonia, particularly when the solution is fresh. For this reason two or three consecutive samples of room or outdoor air should be tested after renewal of the solution, and the apparatus should not be put back in service until the difference between the initial and residual samples comes to about 20 per cent. With this precaution Badger's<sup>3</sup> original solution of half strength ammonia water saturated with ammonium chloride has proved quite satisfactory and it seems to have some advantages over other solutions<sup>1, 4</sup>. As Badger noted volatilization of ammonia from fresh solution, though considerable during the first determination, is usually negligible thereafter. In very hot weather however it has been necessary to add as much as 2 cc. of 20° hydrochloric acid to the solution in the chamber to prevent continued liberation of ammonia gas.

The *setting of the solution* must be such that on introduction of a 10 cc. gas sample into the chamber there will be neither spilling of solution nor escape of gas bubbles. It is convenient to have the surface of the solution in the chamber just below the top of the copper, and the layer of mineral oil covering the column in the reservoir should be between the middle and the lower end of the straight portion of the tube. This difference in the height of the two columns is induced by withdrawing air from the chamber into the syringe while the glass stopcock is open and the metal cock is in position 3, which connecting tube *E* with the outside, shuts off both from the *T* tube. After the levels are set, the glass stopcock may be closed and the syringe emptied of air with the metal cock in position 2.

To maintain this setting, the glass stopcock must not be opened when the metal cock is in position 1 or 2. If such an accident occurs, the glass cock is shut, and any gas in the syringe is ejected to the outside with the metal cock in position 2. The metal cock is then turned to position 3, the glass stopcock is opened, and the plunger is moved back and forth several times to allow complete absorption of the oxygen in the system. The levels are then reset.

*Oxygen Determinations*—The procedure differs but little from that of other bedside apparatus. The rubber tube is connected with the tent outlet valve or introduced under the hood. A small gap in the zipper of one of the newer hoods makes possible an almost airtight connection. After repeated rinsing of the tube by the procedure already described, the sample for analysis is taken by withdrawing the plunger precisely to the 10 cc. mark. The plunger is held at this mark while the metal cock is turned from position 1 to position 3, and the glass stopcock is opened for an instant, then shut. A shift of the oil meniscus greater than the equivalent of about 0.2 cc. indicates that the analysis should be abandoned, but such shifts have been obtained only when the sample had been taken from a source in which the pressure was markedly greater or less than atmos-



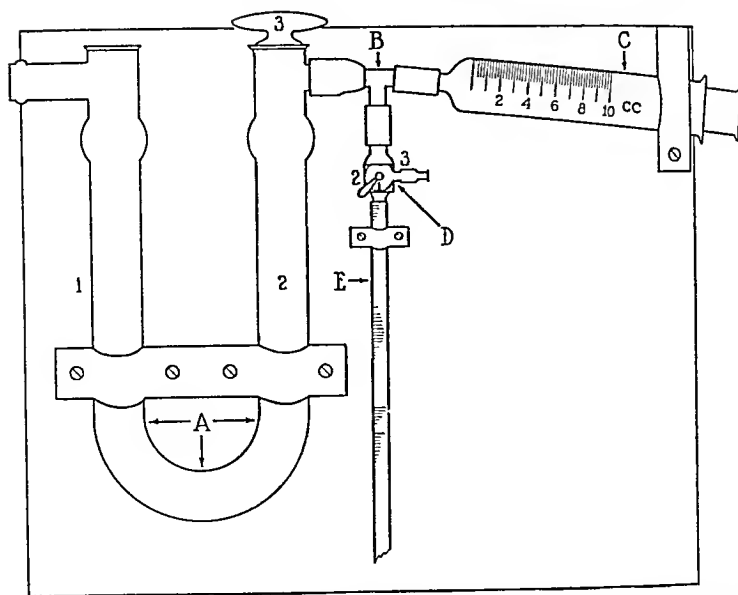


FIG. 1.—A, U-tube; 1, reservoir side; 2, absorption chamber side (contains copper gauze); 3, handle of glass cock. B, T-tube. C, Syringe. D, Three-way metal stopcock; 1, setting of handle for position No. 1; 2, setting for position No. 2; 3, setting for position No. 3 E, Rubber tubing from tent

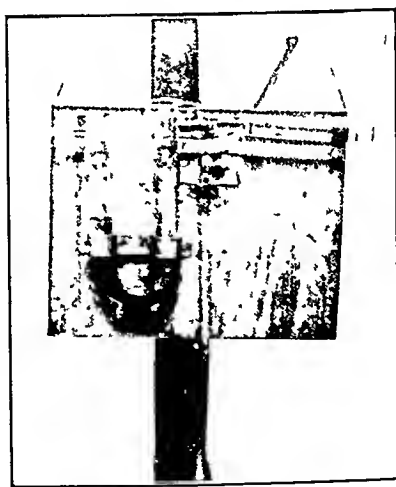


FIG. 2

pheric, never with tent samples. With the not infrequent slight shifts the thread tied around the reservoir tube is simply adjusted from the old to the new level of the oil meniscus. The glass stopcock is again opened as the plunger is pushed all the way in, then promptly shut. This stopcock is not reopened until just before the final reading. Since the rinsing has made the gas in the syringe and T-connection practically homogenous, the air displaced from the T-connection to the chamber as the plunger is first pushed in will be equivalent to the air left in the T-connection when the syringe is emptied. Since this air is not allowed to lose its oxygen, the assumption that deoxygenation affects neither more nor less than a valid 10 e.e. sample involves no significant error. As a matter of fact, the

dead space in our outfits was so small in comparison with the volume of the syringe that repeated moving of air to and from the chamber made only a slight difference in the result. After three minutes have been allowed for absorption, if the fluid hue on the copper has stopped climbing the glass stopcock is opened and the plunger withdrawn until the oil meniscus is brought down to the thread which marked its position before the sample was transferred to the chamber. The position of the plunger now marks the final reading. The calculation  $10 (10 \text{ minus final reading in c.c.}) = \text{percentage of oxygen}$ . If desired a per cent scale may be marked on a strip of adhesive tape beside the syringe. After the reading, the top of the copper may be washed off by withdrawing the plunger a little further. It is best not to bring the solution quite up to the bottom of the glass cock, for lubricant, if picked up repeatedly by the solution might eventually coat much of the copper and hinder oxygen absorption. The solution is reset, the glass stopcock is closed, and the syringe emptied with the metal cock in position 2.

*Care of the Apparatus*—A year's experience has shown that these outfits are sturdy and require little attention. When the solution begins to show a green precipitate, it should be poured out of the reservoir side after removal of the chamber cock. Occasionally a considerable amount of oil or grease will have accumulated in the chamber. Such material may be floated out by washing the U tube through and through with water. Fresh Badger's solution is poured in through the reservoir side, and the chamber stopcock is dried and lubricated. The column of fluid in the reservoir is covered with fresh mineral oil, and the levels are reset. The syringe and the glass stopcock must be kept lubricated, and occasional aspiration of a little mineral oil through the metal stopcock may be helpful. The Badger's solution should not be allowed to reach the chamber side arm and its connections. Leaks should be searched for occasionally by introducing a little air into the syringe, then turning the metal cock to position 3, and with the glass stopcock closed, first pulling, then gently pushing on the plunger. If the fluid in the U tube does not shift, and the plunger moves back to approximately its previous position on release, a further test may be performed by letting rubber tube *E* hang down into a beaker of water and aspirating water into the syringe air being eliminated from the connections by the usual rinsing procedure.

*Accuracy of the Determinations*—This is limited by the fact that neither the oil meniscus nor the syringe is adapted to precise reading. The error of reading may amount to as much as 2 or 3 per cent oxygen.

Reliability of these determinations within this limit was demonstrated both by analyses of outdoor air and oxygen from a tank and by parallel determinations of tent air with the simple outfit and with the Haldane. The former procedure is the simpler for routine checks. The pure oxygen is most easily obtained through a T tube connection which permits release of excess pressure.

For Haldane analysis tents were used which permitted connection of a rubber tube either through an outlet valve or through a tight fitting gap in a zipper in order that change in the tent air might be minimal during sampling. The specimen for the Haldane was taken with a Collins sampling pump, while the sample for the bedside analyzer could be obtained either from the pump or directly from the tent, provided that in the first case the pump pressure was kept

close to atmospheric pressure during transfer of the sample, and in the second case the end of the rubber tube *E* was placed in the main stream of circulating tent air. Determinations with the bedside analyzer were always made first to determine the gas volume which must be handled in the Haldane in order to have a residual large enough to be measured in the graduated portion of the burette. This volume was always more than 10 c.c., since the oxygen content of the samples was 40 to 65 per cent. It was necessary, therefore, to introduce two or more consecutive samples into the burette. After the first sample was measured, it was promptly transferred in toto to the pyrogallol chamber, the mercury being brought precisely to the top of the burette by adjustment of a pinchcock. The carbon dioxide content of the specimen was then determined on the second sample. After the burette was again connected with the pyrogallol chamber, the leveling bulb was cautiously raised and lowered several times until the gas volume had shrunk so that spilling of pyrogallol or escape of bubbles need no longer be feared. Absorption was then completed in the usual manner with at least two rinsings of the air left in the hydroxide capillaries. Although not capable of the highest precision, this method gave oxygen values within 0.1 per cent of one another on duplicate analysis.

*Limits to precision attainable with any bedside apparatus* are such that determinations with the outfit just described are perhaps as accurate as those obtainable with most others. With all of them the directions call for round number measurement of the initial volume. The setting at which the final reading is to be made is usually that assumed by the ammoniacal solution, as connection is made between the chamber and the sample container (syringe or burette) while the plunger or meniscus of the latter is held at the round figure. Precision demands that as in the Haldane, initial as well as final volume be measured in accordance with a previous setting, and such a measurement does not usually come in round numbers. If the initial and final readings are made at approximately the same pressure, the difference will represent well enough the volume which the absorbed gas had occupied at that pressure, but the original pressure adjustment between chamber and sample container, if appreciable, will invalidate the initial reading. Another source of uncertainty could only be eliminated by separate determination of carbon dioxide, for the copper-ammonia chamber, like other devices for oxygen absorption, absorbs carbon dioxide as well. Thus it was found that two successive samples of 70 per cent oxygen and 30 per cent carbon dioxide were almost completely absorbed in our apparatus. Absorption of several consecutive samples of approximately pure carbon dioxide was likewise complete, even when Badger's solution was replaced by saturated aqueous ammonium chloride. This observation illustrates the well-known ability of moist copper to absorb carbon dioxide<sup>2</sup> and shows that such absorption in an ordinary copper-ammonia chamber need not be attributed solely to the alkaline solution. The use of ammonium carbonate solutions offers no way out of this difficulty, since they tend to give off both carbon dioxide and ammonia, and to inhibit the absorption of oxygen.<sup>1</sup> A third possibility of error, the volatilization of ammonia already mentioned, makes the copper-ammonia chamber unsuitable for research purposes. However, such chambers, with their safety, rapid action, and prolonged usefulness, are not likely to be displaced from bedside work by either pyrogallol or hyposulfite.

*Comparison with other analyzers* suggests that those of Hochstetter<sup>3</sup> and Riesman and Lesnick<sup>4</sup> are slightly more complicated in construction than the one here described. They are equipped with finer scales for measurement of the residual sample, and may be even simpler in operation. The author's prejudices in favor of his own device are based on the readily made connections, the use of the syringe for all measurements which obviates the necessity of a second scale, the ease with which solution or even copper may be renewed, the compactness of the mounting, and the simple construction which requires neither cement nor glass blowing. If others should share these views, this apparatus might attain wider use.

#### REFERENCES

- 1 Bridger, W. L. Determination of Oxygen by the Copper-Ammonia-Ammonium Chloride Reagent, *J. Indust. & Engin. Chem.* 12: 16, 1920.
- 2 Mellor, J. W. *A Comprehensive Treatise on Inorganic and General Chemistry*, London, 1922. Longmans, Green & Co., Vol. 2, pp. 75-80.
- 3 Hochstetter, Stanton S. An Oxygen Analyzer for Use in Oxygen Tent Therapy, *J. Lab. & Clin. Med.* 22: 1082, 1927.
- 4 Riesman, J. E. F., and Lesnick, G. A Simple Method of Oxygen Analysis for Use in Oxygen Tent Therapy, *New England J. Med.* 215: 6, 1936.

## A SIMPLE TECHNIQUE FOR CONCENTRATING TUBERCLE BACILLI IN SPUTUM\*

N. P. SULLIVAN, M.S., AND H. J. SEARS, PH.D. PORTLAND, ORE

ONE hesitates to add a technique for sputum concentration to a literature already oversupplied with methods and modifications. For such a new method to be worth while it should be (1) less expensive (2) more rapid (3) more simple in manipulation, or (4) give better concentration of the tubercle bacilli than methods now in use. We believe that the method to be described fulfills some or all of these requirements and thereby justifies its presentation.

The digestant used in our method is a crude preparation of the enzyme papain which is marketed commercially under the name Caroid by the American Ferment Co., Buffalo, N. Y. Other preparations of papain are on the market and might be quite as efficient. We have not tested any of them.

The method for using the papain digestant is as follows. Approximately 0.5 gm. of papain, either in the powder form or mixed with a small amount of water to form a paste, is mixed with 50 c.c. of sputum. The proportions of enzyme to sputum may be varied considerably without affecting the results. If a large number of sputa are to be concentrated at once it is more convenient to dilute the enzyme with water just before adding it to the sputa. The stock enzyme, however, should always be kept in the powdered form as the solutions do not keep well.

\*From the Department of Bacteriology, University of Oregon Medical School, Portland.  
Received for publication, October 10, 1938.

The enzyme-sputum mixture is incubated at 37° C. in either a water bath or hot-air incubator. As soon as complete liquefaction occurs, the mixture is centrifuged at high speed, and smears are made from the sediment and stained in the usual way. The time required for liquefaction varies with the sputum and the enzyme concentration. The average time is ten to fifteen minutes. Papain digestion gives a creamy sticky sediment that adheres well to slides. Because of this, much thicker smears are possible than by the usual concentration methods. Cellular elements and organisms other than the tubercle bacilli are not destroyed by this method. Because other organisms are not destroyed, if cultures or animal inoculations are to be made further treatment of the sediment is necessary. We found that treating the sediment with either acid or alkali gave satisfactory cultures and animal infections.

Cultures of the powdered enzyme showed it to contain a number of various kinds of organisms. For this reason each batch of enzyme was tested for acid-fast bacilli. In no case were any acid-fast organisms found.

To test the efficiency of the enzyme digestion, 50 sputa were selected that contained from one organism in 10 fields to 10 organisms per field, as determined by direct smear. Each sputum was then divided into equal parts, one-half was digested by 4 per cent sodium hydroxide, the other by the enzyme method. After digestion direct smears were made from the enzyme-digestion mixture before centrifuging to compare these smears with those made directly from the undigested sputum. Smears of the sediment from the enzyme and the sodium hydroxide concentrations were made and all were stained by the Ziehl-Neelsen method. In addition to the methylene blue counterstain duplicate slides were counterstained with a saturated aqueous picric acid solution. Extra thick smears were also tried from both types of concentrated sediment. In the case of the thick smears the picric acid counterstain was by far the better. In addition to centrifuging, chemical flocculation, as advocated by Hanks, Clark, and Feldman,<sup>1</sup> was used on the enzyme-digested sputum. It was found that chemical flocculation with either ferric chloride or potassium alum could be used on enzyme-digested as well as with alkali-digested material.

It was found that sputum that had been enzyme digested, well mixed and smeared, gave a higher average number of organisms per field and a much more even distribution of organisms than by direct smear from undigested sputum. In many cases the smears from the digest gave twice as many organisms per field as did the direct smears. In the case of the centrifuged enzyme-digested sputum, the number of organisms per field was practically the same as by the alkali digested in 90 per cent of the sputa. In 10 per cent the enzyme digestion method was definitely superior. Following are a few illustrative counts:

	Number of Organisms per Field in Duplicate Specimens			
Papain digestion	101	79	99	40
Sodium hydroxide digestion	90	58	83	31
				55
				42

*Cultivation and Animal Inoculation of Material Digested by Papain.*—Twelve sputa were selected that were positive for acid-fast bacilli in enzyme-digested sediment. The sediments were treated with either acid or alkali. Cultures were made on Corper's and Petroff's media and guinea pigs were inocu-

lated. Positive cultures were obtained from all of the sputa, and autopsies of the guinea pigs showed many typical lesions in all of the animals.

*Effect of Enzyme Digestion on Organisms Other Than Tubercle Bacilli*—The sediments from all of the enzyme digested sputa were stained by Gram's method and by Churchman's capsule stain. In all cases the Gram stain appeared typical, and in a few specimens encapsulated organisms were demonstrated by the Churchman method. A number of organisms in pure culture were then submitted to the action of the enzyme. *Hemolytic streptococcus*, *E. typhi*, *S. para typhi*, *Esch. coli*, *D. pneumoniae*, *C. diphtheriae*, and *C. hofmanni* strains were grown on Douglas chocolate agar for twenty-four hours, the growth emulsified in 5 cc of saline papain solution and incubated for three hours at 37° C. Streaks were then made from these on Douglas blood agar and Douglas chocolate agar plates. In every case the specified organism was recovered. As stated previously, the enzyme powder is not sterile, so the streak plates contained organisms other than those under test. No difficulty was encountered, however, in isolating the test organism.

#### CONCLUSIONS

An enzyme digestion method for concentrating the tubercle bacilli in sputum has been described which has the following advantages:

- 1 Digestion of sputum is rapid, usually being complete within ten to fifteen minutes.
- 2 No neutralization of the digestant is required.
- 3 The sediment obtained from the enzyme digestion adheres well to glass slides, allowing thicker smears.
- 4 Cultures and animal inoculations may be made from the sediment after appropriate treatment to destroy nonacid fast organisms which are not affected by the digestant.

#### REFERENCE

- 1 Hanks, J. H., Clark, H. F. and Feldman, H. Concentration of Tubercle Bacilli From Sputum by Chemical Flocculation Methods. J. LAB. & CLIN. MED. 23: 736, 1938.

# A MANUAL OF NEUROHISTOLOGIC TECHNIQUE

OSCAR A. TURNER, M.D., NEW HAVEN, CONN.

(Continued from the June issue, page 1003.)

## CHAPTER XI

### MISCELLANEOUS STAINING METHODS

*Iron.*—There are many methods for the microchemical determination of iron in tissues. A simple and rapid procedure is the Perl test which depends upon the formation of a blue color by the action of potassium ferrocyanide upon the iron contained in hemosiderin. The method is sometimes referred to as the Prussian blue reaction.

1. Sections are brought to distilled water. Frozen or celloidin sections may be used, but in the latter the celloidin must be removed before staining.

2. Treat for thirty minutes to one hour in a mixture of 1 part freshly prepared 2 per cent potassium ferrocyanide and 3 parts of 1 per cent hydrochloric acid by weight. The latter is prepared by adding 2 c.c. of concentrated hydrochloric acid to 75 c.c. of distilled water.

3. Wash thoroughly in four to five changes of distilled water.

4. Counterstain lightly with eosin.

5. Dehydrate in alcohols, clear in xylol, and mount in balsam. The iron (hemosiderin) is stained blue upon a pink or red background. The stain is quite intense and very sharp. The best results are obtained upon alcohol or formalin-hardened tissue.

Zenker-fixed tissue may be used, but the blue color is apt to be diffuse. Paraffin, celloidin, or frozen sections may be used. Mallory recommends counterstaining with 0.1 to 0.5 per cent basic fuchsin in 50 per cent alcohol. With this counterstain the nuclei and hemofuscin are stained red, and the hemosiderin blue. The preparations tend to fade in several months.

For the rapid detection of iron in brain tissue, especially in suspected cases of dementia paralytica, the Spatz reaction is useful. Material from the frontal cortex will give the most pronounced results. A small piece of fresh, unfixed cortex is immersed for a short time in physiologic saline solution. It is then transferred to concentrated ammonium sulfide and allowed to remain fifteen minutes. In general paralysis one sees fine, dark streaks and punctate areas in the cortex. By means of a glass hook such an area is removed and brought to physiologic saline. It is then trimmed of as much excess tissue

as possible and transferred to a small drop of glycerin on a glass slide. A cover slip is placed over it, and by firm pressure the tissue is spread flat. Under the microscope one can distinguish greenish black clumps and granules of iron in the vessel walls and cells.

**Calium**—Calcific deposits in tissue stain deeply with hematoxylin so that special methods are usually not necessary. Von Kossa's method is useful when a differential stain for these lime deposits is needed.

- 1 Paraffin, celloidin, or frozen sections of alcohol or formalin hardened tissue are used.

- 2 Place sections in a 1 to 5 per cent solution of silver nitrate for thirty to sixty minutes.

- 3 Wash thoroughly in distilled water.

- 4 Dehydrate in ascending alcohols clear in xylol and mount in balsam.

Calium salts are stained a deep black. The nuclei can be counterstained with alum carmine or safranine after the silver impregnation. The method depends upon the formation of silver carbonate or silver phosphate which, in the presence of light, is converted to black silver oxide. If the sections are left in the silver solution as long as twelve hours there is more assurance that all of the calium phosphate will have been stained.

The sections should be exposed to direct sunlight while in the silver bath. The reaction may be hastened by a few minutes' exposure to a direct source of ultraviolet light.

**Staining Tumors for Rapid Diagnosis**—It is often desirable to obtain a quick histopathologic report upon neoplasms exposed during an operation. The fact that many of the intracranial tumors are ill suited for frozen section procedures makes desirable some method whereby unfixed bits of tissue may be stained rapidly and prepared for microscopic examination.

**Supravital Staining**—This method has been applied by Eisenhardt and Cushing with good results. The method has the disadvantage of requiring familiarity with the appearance of cells stained with a dilute solution of neutral red dye.

As soon as the tumor is exposed at operation a minute fragment is separated and placed on a clean glass slide. A drop of aqueous solution of neutral red dye (1:10,000 or more) is added directly to the tissue. A cover slip is then applied, and by gentle pressure the tissue is spread. The preparation is rinsed with a paraffin vaseline mixture and examined immediately under the microscope in a warm box.

The amount of pressure required to obtain an even preparation varies with the type of tissue and is learned only by experience. If the solution of the dye is too strong, the nuclei will stain diffusely, a sign that the cells have been injured, or the tissue will be overstained, making the diagnosis difficult. It is important that the preparation be made as soon as possible after the tissue has been removed. Tissue which has been allowed to dry is useless, but stained preparations retain their clarity for an hour or more if kept in the



warm box and if drying is prevented. Upon standing there is a tendency for the cells to become vacuolated and for the cytoplasm to become granular.

The microscopic appearance of each of the various types of intracranial tumors when stained in this manner can be found in the original articles by Eisenhardt and Cushing. However, a few characteristics of the more common types will be given. The fibrillary astrocytomas usually appear as a dense network of neuroglia fibers, which are rarely stained but which appear as highly refractile processes with scattered nuclei. In the less dense areas along the margins of the preparation an unmistakable astrocyte with long processes may be seen. Occasionally there is an abundance of astrocytes. In tumors which are composed predominantly of the protoplasmic astrocytes there are few fibrillae and, if the specimen is not too degenerate, astrocytic cells may be seen. Tissue from glioblastomas shows cells of various shapes and forms, astrocytes, spongioblasts, and multinucleated forms. There may be many mitotic figures. In some instances, if the tissue is necrotic, the field may consist of large, vacuolated cells filled with refractile globules having a characteristic greenish-red color. Medulloblastomas appear as closely packed round cells with pale but well-defined cytoplasm. Mitotic figures are abundant. The cells of the oligodendrogliomas are also well defined. The nuclei are spherical, contain considerable chromatin, and are surrounded by an abundance of pale cytoplasm. Mitotic figures are not seen. The meningiomas are easily recognized by the characteristic whorl formation. The cells have large oval nuclei and are usually well defined.

It should be noted that if nervous tissue from the immediate region of a tumor is examined, an unusual number of neuroglia fibrillae and astrocytes may be observed. A representative piece of tissue is, therefore, necessary for diagnosis. The recognition of characteristic myelin sheaths will often differentiate a reactive gliosis from actual tumor tissue.

*Azure A-Erie Garnet B Stain.*—This method, described by Deery, has been found useful for rapid diagnosis and requires no special experience.

A small piece of tumor, trimmed free from all blood clot and apparent non-neoplastic tissue, is transferred to a new, clean glass slide. With two dissecting needles the tissue is teased into small fragments or a thin layer, depending upon its consistency. A few drops of the dye mixture of azure A and Erie garnet B are then added, and the preparation is allowed to stand about fifteen seconds. Without washing off the excess stain a cover glass is applied and the preparation is firmly pressed between two blotters. It is then examined under the microscope.

The preparation of the staining solution has been described by Geschickter. To 4 parts of a previously filtered 1 per cent aqueous solution of azure A, there is added 1 part of a filtered 0.5 per cent aqueous solution of Erie garnet B. The latter is added quickly and the resultant mixture is filtered immediately to prevent precipitation. The mixture keeps well, but it may be necessary to refilter after it has been standing for several weeks.

For frozen sections Geschickter recommends staining for ten to fifteen minutes in the dye mixture, washing in two changes of distilled water and mounting in 40 per cent glucose. After the second wash, the section is trans-

ferred to a clean slide where a cover slip having upon it a large drop of 40 per cent glucose is inverted over the section. The preparation may be examined after allowing a minute or so for clearing to take place. The section should be handled with a glass hook from which it need not be removed until ready for mounting.

The azureA-Erie garnet B method gives a stain which is similar to hematoxylin and eosin, but the final preparation is said to be much clearer and with greater detailed staining. The stain does not fade.

*Stains for Negri Bodies.*—Only occasionally in the average laboratory is there need for a stain for Negri bodies. It is advisable, therefore, that the methods employed use staining solutions which are either quite stable or readily prepared. The tissue examined should be from either the cornu ammonis (hippocampus major) or cerebellum, and if smears or impressions are to be prepared, fresh, unfixed tissue is necessary. If the tissue is to be embedded in paraffin, it should be fixed in alcohol or acetone as soon after death as possible. To prepare an impression, place a small disk of tissue upon a board with one of the cut surfaces up. Press the surface of a clean glass slide upon the disk of tissue and lift it suddenly. If the tissue has not been allowed to dry, it will adhere to the board and leave an impression on the slide. To prepare a smear, place a small piece of tissue on a clean slide. With another slide used as a spreader, press evenly and firmly upon the tissue until it covers the width of the slide. Then draw the spreader toward the opposite end of the glass slide, maintaining an even pressure. The piece of tissue should not be too large, otherwise a thick smear will result.

*Sellers' Method.*—This method has found wide usage but has the disadvantage of requiring undried and unfixed smears or impressions.

The preparations, while still moist with the tissue juices, are plunged into the staining solution and immediately removed. They are then washed in tap water and allowed to dry in the air. The staining solution is prepared in the following manner:

Basic fuchsin (saturated absolute methyl alcoholic solution)	2 to 4 c.c.
Methylene blue (saturated absolute methyl alcoholic solution)	15 c.c.
Methyl alcohol (absolute, acetone free)	25 c.c.

The methylene blue solution and methyl alcohol are mixed in a Coplin jar and 2 c.c. of the basic fuchsin solution are added. A trial smear is made. Macroscopically, the properly stained smear should appear reddish violet in the thinner portions. If on trial smears the thinner portions are blue, more of the basic fuchsin is added. Two cubic centimeters are all that is usually required. The mixture improves after standing for twenty four hours, and keeps indefinitely if evaporation is made up with additional methyl alcohol. Evaporation causes the fuchsin stain to become dominant. The staining solution should be kept in a tightly stoppered bottle.

The Negri bodies are stained a bright, cherry red, and the basophilic or blue staining masses are quite clear. The cytoplasm of the nerve cells is a

purplish blue, the nuclei a deep blue. Nerve fibers are stained a deep pink and bacteria, if present, an intense blue. Erythrocytes appear copper colored. Myelin sheaths are not stained.

While the best results are obtained from fresh tissue, the Negri bodies often may be recognized in degenerated areas. The method is useful for smears of pus and exudate and stains many forms of protozoa, especially the flagellates and ciliates. However, smears which have been allowed to dry or which have been fixed in alcohol, formol, or Zenker's solution, or any other fixative cannot be used. Sections of tissue cannot be stained by this method. It is important that the methyl alcohol be absolutely free from xylol and that the smear be moist when plunged into the stain. Staining should not exceed a few seconds, since even after thirty seconds the results are practically useless.

*Mann-Böhne Method.*—The method is applicable to smears or sections. The tissue should be fixed in alcohol or acetone and embedded rapidly in paraffin.

1. Stain thin sections for one-half to four minutes in the following mixture:

1% aqueous methylene blue	35 c.c.
1% aqueous cosin	35 c.c.
Distilled water	100 c.c.

2. Wash briefly in water.

3. Wash in equal parts of absolute alcohol and water.

4. Allow sections to remain for fifteen to thirty seconds in alkaline alcohol. This is prepared by the addition of 5 drops of 1 per cent sodium hydroxide in absolute alcohol to 30 c.c. of absolute alcohol.

5. Wash briefly in absolute alcohol.

6. Wash in tap water.

7. Transfer to tap water which has been made acid by the addition of 1 drop of acetic acid to 30 c.c. of water. Allow to remain one to two minutes.

8. Wash rapidly in absolute alcohol and clear in xylol. Mount in balsam.

This is an excellent stain provided the sections are not overdecolorized in the alkaline alcohol. The Negri bodies are seen as small oval bodies stained a deep pink, lying within the pale blue cytoplasm of the nerve cells. There is often a more deeply stained peripheral zone with one or more small vacuole-like bodies within it.

In addition to the above two methods, excellent preparations may be obtained upon embedded material by the method of Lentz, or upon smears or impressions by the methods of Frothingham, van Gieson, and Giemsa.

*Gross Staining of the Brain.*—Practical museum preparations, especially useful for the teaching of anatomy or pathology, may be obtained by the macroscopic staining of sections of the brain. Such preparations, when properly stained, give sharp contrast between gray and white matter and can be

kept indefinitely for display or class use. The following method, described by Le Masmnier, has been found to be the most dependable.

1 The brain should be thoroughly hardened in formalin. Slices are cut to the desired thickness with a knife which is well lubricated with glycerin. The sections must be cut in one stroke to avoid leaving knife marks. They should not be handled with the fingers.

2 Wash the specimens from twelve to twenty-four hours in running water. Transfer to distilled water and allow to remain for one hour, changing the water at least three times during this period.

3 Submerge the sections for two minutes in Mulligan's solution of phenol at from 60° to 65° C. There should be enough of this solution to cover the sections by 2 to 3 inches. The formula for Mulligan's solution is as follows:

Phenol crystals	40.00 gm
Concentrated hydrochloric acid	1.25 cc
Copper sulfate (crystals)	5.00 gm
Distilled water	1000 cc

4 Submerge in an abundance of cold tap water for one minute.

5 Transfer to a 1 per cent solution of ferric chloride in distilled water and allow to remain two minutes.

6 Place in a 1 per cent aqueous solution of potassium ferrioxalate until the gray matter is a brilliant blue. This should take no longer than three minutes.

7 Wash in running water for twenty-four hours.

8 Preserve sections in 70 per cent alcohol.

The cortex and central nuclei, such as the caudate, thalamus, and dentate of the cerebellum, are stained a brilliant blue. Areas of demyelination and degeneration are also stained blue as are intra-axial tumors. The white matter remains a creamy white. The preparations may be exposed to direct sunlight without fading. It is very important that the knife used to cut the brain be well lubricated with glycerin and that the slice be cut in one stroke. Failure in this respect will result in diffuse coloration of the white matter. Sections should be handled as little as possible and then with great care. Excessive and rough handling of the slices will cause the white matter to assume a blue color.

*Histotopographic Methods*—Largely through the work of Christeller, a technique has been devised for the preparation of frozen sections of complete organs. These preparations are suitable for microscopic study. The method is of particular value in that it allows for both a topographic and a microscopic survey of a cross section of a complete organ. The microtome used for the purpose of making these large frozen sections is of a special type with a large freezing chamber and double fixation of the knife to prevent vibration. For the exact technique the reader is referred to the Foreword in Dr. Christeller's Atlas or to the article by Dr. Maude E. Abbott.

## CHAPTER XII

## FORMULAS

*Zenker's Fixative.*

Potassium bichromate	2.5 gm.
Mercuric chloride	5.0 gm.
Water	100.0 c.c.
Glacial acetic acid	5.0 c.c.

Dissolve the potassium bichromate and mercuric chloride in the water with the aid of heat. Add the glacial acetic acid in the proper proportion just before use. Tissue should be fixed in this fluid for ten to twelve hours. Prolonged fixation produces crystals of mercuric chloride which are difficult to remove. Sections of tissue which has been fixed in Zenker's fluid must be treated with Gram's iodine and 5 per cent sodium thiosulfate before staining to remove the crystals which are deposited.

*Regaud's Fixative.*

5% potassium bichromate	3 parts
Commercial formalin	1 part

The formalin is neutralized with magnesium carbonate. The solution should be changed daily. This is the best fixative for pituitary tissue and for certain of the nonmetallic glial stains.

*Orth's Fixative.*

Potassium bichromate	2.5 gm.
Water	100.0 c.c.
40% formalin	10.0 c.c.

This is a good general fixative. It is improved by the addition of 5 per cent acetic acid just before use.

*Müller's Fluid.*

Potassium bichromate	2.5 gm.
Sodium sulfate	1.0 gm.
Distilled water	100.0 c.c.
Heat together until dissolved.	

This is a slowly acting fixative valuable for nervous tissue. The solution should be changed frequently and kept in the dark to prevent precipitation.

*Cajal's Formalin-Ammonium Bromide.*

Formalin (Merek's blue label, 40%)	14. c.c.
Ammonium bromide	2 gm.
Distilled water	86 c.c.

This fixative is used particularly for the demonstration of the interstitial cells of the nervous system by the metallic impregnation methods. Tissue should not remain in this solution for more than six to seven days.

*Formol Saline.*

Sodium chloride	1 gm
Formalin	10 cc
Water, to make	100 cc

This can be used as a routine fixative in place of 10 per cent formalin Addison, in McClung's *Handbook*, recommends the addition of sufficient sodium chloride to 5 per cent formalin solution to bring the specific gravity to 1.030. He states that in this solution there is less initial change and that the brain floats, submerged in the solution, thus preventing distortion by the sides of the container.

*Kaiser's Glycerin Jelly*

Finest French gelatin	8 gm
Phenol crystals	5 gm
Glycerin	50 cc
Distilled water	42 cc

Mix the glycerin and gelatin together and place in a water bath at 55° C until the gelatin is completely dissolved. Add the phenol crystals and filter. Allow to cool and harden. Melt in a bath of boiling water before use. The gelatin may be dissolved first in the water, and the phenol and glycerin added later. The solution must be filtered through glass wool. If coarse filter paper is used, the process must be carried out in an incubator and requires eighteen to twenty-four hours.

*Arabic Sugar*

Pure gum arabic	20 gm
Sucrose	20 gm
Distilled water	50 cc

Dissolve the sugar in the water. Rub the gum arabic until finely pulverized and add slowly, avoiding the formation of bubbles as much as possible. Add a few crystals of phenol and stopper tightly. Heat in an oven until thoroughly dissolved. The solution should be clear. This medium has a refractive index the same as balsam. It sets quite hard and can be used where sections must be mounted directly from water.

*Delafield's Hematoxylin*

Hematoxylin crystals	4 gm
Absolute alcohol	25 cc
90% alcohol	100 cc
Glycerin	100 cc
Saturated aqueous solution of ammonia alum in distilled water	400 cc

Dissolve the hematoxylin in the absolute alcohol and add the saturated ammonia alum solution. Mix well and allow to stand open, exposed to direct sunlight if possible, for three to four days. Filter and add the glycerin and 90 per cent alcohol. The mixture should be diluted considerably with distilled water before using. About 30 to 40 gm of ammonia alum are required.

to saturate 400 c.c. of water. This is a powerful nuclear stain which keeps well. It may be necessary to ripen the solution for a long period to obtain the optimum results.

*Ehrlich's Hematoxylin.*

Hematoxylin crystals	2 gm.
96% alcohol	100 c.c.
Distilled water	100 c.c.
Pure glycerin	100 c.c.
Potassium alum	3 gm.
Glacial acetic acid	10 c.c.

Dissolve the hematoxylin in the alcohol and add the other ingredients, leaving the acetic acid to the last. The solution should be a light red when first mixed, but should darken on exposure to light. It should be placed in a cotton-stoppered glass flask and exposed to direct sunlight for at least fourteen days. Better results are obtained after exposure to light for six months. About 0.5 gm. of hematin may be used instead of the hematoxylin, in which case the solution may be used sooner. The nuclei stain a clear blue. After staining the section should be washed thoroughly.

*Mayer's Hematoxylin (Hemalum).*

Hematoxylin	1.0 gm.
Distilled water	1000.0 c.c.
Sodium iodide	0.2 gm.
Potassium alum	50.0 gm.

This is an improvement over the original Mayer formula. The hematoxylin may be first added to 50 c.c. of 90 per cent alcohol and dissolved with the aid of heat. The water and alum are then added. The solution acts much as Delafield's hematoxylin, but does not keep as well. A crystal of thymol may be added as a preservative. The addition of 2 per cent glacial acetic acid to this stain constitutes Mayer's acid alum. The acid preparation gives a clearer nuclear stain than does the above formula.

*Weigert's Iron Hematoxylin.*

A. Hematoxylin	1.0 gm.
96% alcohol	100.0 c.c.
B. Ferric chloride	0.4 gm.
Distilled water	100.0 c.c.
Hydrochloric acid	1.0 c.c.

Equal parts of these two solutions are mixed just before staining. Although the mixture keeps for some time, it is best to prepare it fresh. Decolorization may be carried out in a weak solution of ferric chloride.

In certain staining methods, especially those in which strongly acid or alkaline solutions or prolonged treatment in silver salts is used, there may be a tendency for paraffin sections which have been mounted by the glycerin-egg albumen method to float off the slide. If possible, the sections should be mounted with gelatin. However, for routine purposes, the following method

has been useful in preventing detachment. The unstained sections, following treatment with several changes of xylol and hydration through descending alcohols, are brought to water and then to a Coplin jar containing 1 to 3 per cent gelatin solution. They are allowed to remain here at least four hours and as long as twelve hours. Following this they are transferred to 10 per cent formalin without washing and allowed to remain for a similar length of time. Staining is carried out as usual following washing in tap and then distilled water. Provided the sections are not handled roughly, they will adhere to the glass slides.

Sections which are treated with the Weigert mordants before staining should be passed through the gelatin and formalin as outlined above. The method does not interfere with the staining properties of the tissue.

#### APPENDIX

*A Nitrocellulose as an Embedding Agent*—Nitrocellulose may be used as an embedding agent in place of the more expensive celloidin. Since this agent is an explosive when dry, it is usually supplied thoroughly moistened with alcohol. The alcohol content of the material must be taken into account in the preparation of the solution.

The tissue to be embedded after having been dehydrated in alcohols of ascending strengths, is passed through alcohol-ether solution (1:1) and allowed to remain for a period of time in 6 per cent and finally 12 per cent nitrocellulose. The length of time required for complete infiltration of the tissue to take place varies with the size of the tissue block and the concentration of the nitrocellulose solution and must be gauged accordingly. After the tissue has been thoroughly infiltrated it is embedded in fresh 12 per cent solution. However, it may also be allowed to stand for some time in 25 per cent nitrocellulose and embedded in this solution. The final method of hardening the block and sectioning does not differ from that used with celloidin. As with celloidin, the hardening should take place slowly and should not be carried to an extreme.

The following table gives the proportion of the ingredients used in the preparation of approximately 560 c.c. of nitrocellulose solution.

	25% Solution	12% Solution	6% Solution
Nitrocellulose	200 gm	100 gm	50 gm
Absolute alcohol	205 c.c.	245 c.c.	210 c.c.
Ether	240 c.c.	280 c.c.	280 c.c.

The 25 per cent solution may give rise to difficulties in embedding because of entrapped air bubbles. The 12 per cent solution may be prepared by diluting the 25 per cent solution with an equal part of absolute alcohol-ether solution. Similarly, the 6 per cent solution may be prepared by diluting the 25 per cent solution with 3 parts of absolute alcohol-ether solution or the 12 per cent solution with an equal part of absolute alcohol-ether solution. The 6 per cent and 12 per cent solutions may also be prepared from the dried and finely chopped



scraps of nitrocellulose obtained in trimming the embedded blocks. The proportions used here are as for any solution.

Although the time required to embed tissue is slightly longer than with celloidin, the results are equally as good and far less expensive.

*B. Dye Solubilities.*—The following table lists the solubilities for various dyes in both distilled water and 95 per cent alcohol, as given by the Stain Commission. The figures represent the number of grams of stain in 100 c.c. of a saturated solution at 26° C.

<i>Dye</i>	<i>Water</i>	<i>95% Alcohol</i>
Picric acid	1.18	8.96
Orange G	10.86	0.22
Safranine	5.45	3.41
Cresyl violet	0.38	0.25
Thionine	0.25	0.25
Toluidine blue	3.82	0.57
Sudan III	0.00	0.15
Sudan IV (scharlach R)	0.00	0.09
Crystal violet, chloride	1.68	13.87
Eosin, sodium salt	44.20	2.18
Eosin B	39.11	0.75
Erythrosin	11.10	1.87
Methylene blue, chloride	3.55	1.48
Neutral red	5.64	2.45
Aniline blue (spirit soluble)	0.00	1.10

*C. Specific Gravity of Ethyl Alcohol-Water Mixtures.*—A check may be had on the alcohol content of alcohol-water mixtures by determining the specific gravity. This is a valuable and accurate method of determining whether the alcohol used in the final dehydration of sections contains small quantities of water. The following table gives the specific gravity of mixtures of ethyl alcohol and water at 15.56° C., referred to water at the same temperature (U. S. Department of Agriculture).

<i>Specific Gravity</i>	<i>% Alcohol by Volume</i>	<i>Specific Gravity</i>	<i>% Alcohol by Volume</i>
0.98660	10.0	0.8639	80.0
0.97608	20.0	0.8339	90.0
0.96541	30.0	0.8161	95.0
0.95185	40.0	0.8121	96.0
0.9344	50.0	0.8079	97.0
0.9136	60.0	0.8035	98.0
0.8900	70.0	0.7989	99.0
		0.7939	100.0

#### REFERENCES

##### *General*

- Anderson, J.: How to Stain the Nervous System. A Laboratory Handbook for Students and Technicians, Edinburgh, 1929, E. & S. Livingstone.
- Kernohan, J. W.: A New Modification of Mallory-Heidenhain's Differential Staining Method and Adaptation of Formalin-Fixed Material for Mallory's Stains, *Am. J. Clin. Path.* 1: 399, 1931.
- : Adaptation of Formalin-Fixed Tissue to Mallory's Phosphotungstic Acid Hematoxylin Stains, *J. Tech. Methods* 13: 82, 1934.

- Mallory, F B, and Wright, J H *Pathological Technique A Practical Manual for Workers in Pathological Histology and Bacteriology*, Philadelphia, 1938, W B Saunders Co
- Mann, G *Physiological Histology, Methods and Theory*, Oxford 1902, Clarendon Press
- Schmorl, W *Die Pathologisch Histologischen Untersuchungsmethoden*, Leipzig, 1928, F C Vogel
- Spelmeyer, W *Technik der mikroskopischen Untersuchung des Nervensystems*, Berlin, 1930, Julius Springer
- Wertham, R, and Wertham, F *The Brain as an Organ, Its Postmortem Study and Interpretation*, New York, 1934, Macmillan Co

### Nerve Cells

- Bean, R J *Stain Technique for Nissl's Granules Following Alcohol Formal Fixation*, *Stain Technol* 2 56, 1927
- Emerson, L A *Method for Progressive Selective Staining of Nissl and Nuclear Substance in Nerve Cells*, *Am J Path* 8 295 1932

### Myelin Sheaths

- Duncan, D *The Marchi Method A Discussion of Some Sources of Error and the Value of This Method for Studying Primary Changes in the Myelin Sheath*, *Arch Neurol & Psychiat* 25 326, 1931
- Pal, J *Ein Beitrag zur Nervenfarbetechnik*, *Med Jahrb* 82 619 1886
- Smith, W K, and Quigley, B A *New Method for the Rapid Staining of Myelin Sheaths*, *Am J Path* 13 491, 1937
- Stewart, R J C *A Short Marchi Method*, *J Path & Bact* 43 339, 1936
- Weil, A *A Rapid Method for Staining Myelin Sheaths*, *Arch Neurol & Psychiat* 20 392, 1928

### Neurofibrils

- Beech, R H, and Davenport, H A *The Bielschowsky Staining Technique A Study of Factors Influencing Its Specificity for Nerve Fibers*, *Stain Technol* 8 11, 1933
- Bielschowski, M *Eine Modifikation Meines Silberimprägnationsverfahrens zur Darstellung der Neurofibrillen*, *Jahrb f Psychol u Neurol* 12 135 1908
- Bodran, D *A New Method for Staining Nerve Fibers and Nerve Endings in Mounted Paraffin Sections*, *Anat Rec* 65 89, 1936

### Fat Stains

- Rettie, R *A New Method of Applying Nile Blue as a Fat Stain*, *J Path & Bact* 34 595, 1931

### Nonmetallic Glia Stains

- Holzer *Über eine neue Methode der Gliafärbung*, *Ztschr f d ges Neurol u Psychiat* 69 354, 1921
- Mallory, F B *A Contribution to the Classification of Tumors*, *J M Research* 13 113, 1905
- Mann, G *Über die Behandlung der Nervenzellen für experimentell-histologische Untersuchungen*, *Ztschr f wissenschaftl Mikr* 11 479 1894
- Warkany, J *Zur Methodik der Gliafärbung*, *Ztschr f wissenschaftl Mikr* 41 508, 1924

### Metallic Glia Stains

- Corten, M H *Eine Modifikation der Cajal'schen Gold Sublimat Methode zur Darstellung Makrogliazellen am formolfixierten Material*, *Contribl f allg Path u path Anat* 50 339, 1931
- Davidoff, L M *Stain for Fibrillary Neuroglia in Formalin Fixed Material*, *Am J Path* 4 493, 1928
- Dieterle, R R, and Neuman, M *A New Demonstration of Hortega Cells*, *Arch Neurol & Psychiat* 24 1154, 1930
- Fincher, E F, Jr *The Impregnation of Glomer Sections From Paraffined Tissue With Hortega's Silver Carbonate (Lithium) Solution*, *Am J Cancer* 16 918, 1932
- Foot, N C *Comments on the Impregnation of Neuroglia With Ammoniacal Silver Salts*, *Am J Path* 5 223, 1929
- Globus, J H *The Cajal and Hortega Glia Staining Methods A New Step in the Preparation of Formaldehyde Fixed Material*, *Arch Neurol & Psychiat* 18 263, 1927
- Kanzler, R *Eine Modifikation der Darstellung der Hortega'schen Gliazellen für Formalin material*, *Ztschr f d ges Neurol u Psychiat* 122 416, 1929
- Loughlin, E H *A Simple Routine Procedure for Staining Neurons and Glia of the Nervous System*, *Arch Neurol & Psychiat* 33 616, 1935
- Penfield, W *A Method of Staining Oligodendroglia and Microglia (Combined Method)*, *Am J Path* 4 153, 1928
- , and Cone, W V *Neuroglia and Microglia (The Metallic Methods)* in McClung, C E *Handbook of Microscopical Technique*, New York, 1937, Paul B Hoeber

- Pilcher, C., and Owens, E.: A Stain for Neuroglia in Formaldehyde-Fixed Paraffin Sections, *Arch. Neurol. & Psychiat.* 35: 351, 1936.
- Wilder, H. C.: Silver Impregnation of Glia and Nerve Fibers in Paraffin Sections After Formalin Fixation, *Am. J. Path.* 8: 785, 1932.

#### *Connective Tissue Stains*

- Bailey, P., and Hiller, G.: The Interstitial Tissues of the Central Nervous System, *J. Nerv. & Ment. Dis.* 59: 337, 1924.
- Gordon, H., and Sweets, H. H.: A Simple Method for the Silver Impregnation of Reticulum, *Am. J. Path.* 12: 545, 1936.
- Masson, P.: Some Histological Methods, Trichrome Staining and Their Preliminary Technique, *J. Tech. Methods* 12: 75, 1929.
- Perdrau, J. R.: The Silver Reduction Method for the Demonstration of Connective Tissue Fibers, *J. Path. & Bacteriol.* 24: 117, 1921.
- Wilder, H. C.: An Improved Technique for the Silver Impregnation of Reticulum Fibers, *Am. J. Path.* 11: 817, 1935.

#### *Pituitary and Pineal Stains*

- Bailey, P.: Cytological Observations on the Pars Buccalis of the Hypophysis Cerebri of Man, Normal and Pathological, *J. M. Research* 42: 349, 1921.
- Crooke, A. C., and Russell, D. S.: The Pituitary Gland in Addison's Disease, *J. Path. & Bact.* 40: 255, 1935.
- Hortega, P. del Rio: Constitución histológica de la glandula pineal, *Arch. de Neurobiol.* 3: 359, 1922.
- Sparks, C.: A Simple Differential Stain for the Human Hypophysis, *J. LAB. & CLIN. MED.* 20: 508, 1935.

#### *Spirochete Stains*

- Dieterle, R. R.: Method for Demonstration of Spirochaeta Pallida in Single Sections, *Arch. Neurol. & Psychiat.* 18: 73, 1927.
- Nieto, D.: Über ein einfaches Verfahren zur Darstellung von Spirochäten in einzelnen Schnitten, *Klin. Wchnschr.* 12: 1775, 1933.
- Steiner, G.: Über eine neue Spirochätenfärbung im Gefrierschnitt, *München. med. Wchnschr.* 69: 121, 1922.

#### *Spatz Method for Iron*

- Stiefler, G.: Über die Spatzsche Methode der anatomische Schnelldiagnose der progressiven Paralyse, *München. med. Wchnschr.* 70: 704, 1923.
- : Über die Spatzsche Method der histologischen Schnelldiagnose der progressiven Paralyse, *Ztschr. f. d. ges. Neurol. u. Psychiat.* 89: 438, 1924.

#### *Rapid Diagnosis Methods*

- Eisenhardt, L.: Diagnosis of Intraerianial Tumors by Supravital Technique. Further Studies, *Arch. Neurol. & Psychiat.* 28: 299, 1932.
- , and Cushing, H.: Diagnosis of Intracranial Tumors by Supravital Technique, *Am. J. Path.* 6: 541, 1930.
- Deery, E. M.: The Neurosurgical Laboratory, *Bull. Neurol. Inst. New York* 5: 127, 1936.
- Geschickter, C. F.: Fresh Tissue Diagnosis in the Operating Room, *Stain Technol.* 5: 81, 1930.

#### *Negri Bodies*

- Sellers, T. F.: A New Method for Staining Negri Bodies of Rabies, *Am. J. Public Health* 17: 1080, 1927.

#### *Macroscopic Staining*

- Le Masurier, H. E.: Simple Method of Staining Macroscopic Brain Sections, *Arch. Neurol. & Psychiat.* 34: 1065, 1935.

#### *Histotopographical Methods*

- Abbott, M. E.: An Appreciation of Erwin Christeller's Atlas of the Histotopography of Normal and Diseased Organs, With an Exposition of His Technique, *J. Tech. Methods* 12: 105, 1929.
- Christeller, E.: A New Simple Method for the Normal and Pathological Histotopography of Organs, *J. Tech. Methods* 11: 28, 1925.
- : Atlas der Histotopographie gesunder und erkrankter Organe, Leipzig, 1927. Georg Thieme.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A KILDUFFE, M D ABSTRACT EDITOR

**COLD VACCINES**, Evaluation of, Diehl H S, Baker, A B, and Cowan, D W J A M A 111 1168, 1938

In a carefully controlled study of the value of three different vaccines which are recommended for the prevention of colds the subjects were cold susceptible students at the University of Minnesota

A "control group" was observed during each year of the study Such groups were chosen at random from the students who applied for cold prevention treatment, the members were treated in exactly the same manner as those of the vaccinated group, and they believed throughout the period of the experiment that they were receiving vaccine Sterile physiologic solution of sodium chloride was administered hypodermically as a control for the subcutaneously administered vaccine and lactose filled capsules as a control for the vaccines administered orally

One of the most significant aspects of this study is the great reduction in the number of colds which the members of the control groups reported during the experimental period as compared to the number that the same students reported for the previous year In fact, these results were as good as many of those reported in uncontrolled studies which recommend the use of cold vaccines

The group which received vaccine subcutaneously experienced an average of 25 per cent less colds per person than did the control group This difference occurred during both years of the study and is statistically significant Practically, however, it is of little or no importance, because a reduction of 25 per cent in the average number of colds in a group of individuals is not sufficiently great to justify the time and expense involved in carrying out the intensive vaccination procedure which was utilized

The group which received the polyvalent vaccine administered orally experienced just as many colds as the control group during both years of the study

The results reported by the students who took Rosenon's streptococcus vaccine parallel exactly those reported for the control group

Although the data are not entirely conclusive there is no evidence in this study either that vaccines reduce the complications of colds or that the condition of the nose and throat is related to the frequency of colds in a cold susceptible group

**DIPHTHERIA TOXOID**, Reactions Following Use of, Hayman C R Am J Dis Child 56 723, 1938

Reactions to the injection of undiluted plain toxoid are frequent and severe enough to warrant caution

The age of the subject is a good guide in foretelling reaction

The diluted toxoid test is of value in forecasting reaction in those younger than 15 years, the Schick control test is not Both are of value in those older than 15 years

Reactions are less influenced by change in dose of undiluted toxoid than by variation in age and cutaneous sensitivity

Lack of reaction to an initial injection does not warrant giving a much stronger second injection

The following scheme of immunization is suggested

A If the subject is 15 years of age or older

1 Perform preliminary cutaneous tests, using

(a) Schick and Schick control test or

(b) Schick and diluted toxoid (1:20) tests or

(c) all three

2. If the Schick test is positive, give the first injection as follows:

(a) If the Schick control test, the diluted toxoid test or both are negative, inject 0.5 c.c. of undiluted toxoid.

(b) If the highest reading is 1 plus, inject 0.25 c.c. of undiluted toxoid.

(c) If the highest reading is 2 plus, inject 0.1 c.c. of undiluted toxoid.

(d) If the highest reading is 3 plus, inject diluted toxoid or another material, such as toxoid-antitoxin floccules, or do not immunize.

3. For subsequent injection:

(a) If no reaction has occurred, give the same or, at most, a double dose.

(b) If a reaction has occurred, give the same or a decreased dose.

B. If the subject is under 15 years of age:

1. Do the preliminary cutaneous tests, using

(a) Schick test alone or

(b) Schick and diluted toxoid tests.

2. If the Schick test is positive, give the first injection as follows:

(a) If the diluted toxoid test was omitted, inject 0.1 c.c. of undiluted toxoid.

(b) If the diluted toxoid test reading is 1 plus, inject 1 c.c. of undiluted toxoid.

(c) If the diluted toxoid test reading is 2 plus, inject 0.5 c.c. of undiluted toxoid.

(d) If the diluted toxoid test reading is 3 plus, inject 0.25 c.c. of undiluted toxoid.

3. For subsequent injection, proceed as in A.

**BLOOD GROUPING, Artificial Concentration of Test Serums in, Hoxworth, P., and Mahoney, E. J. A. M. A. 111: 1554, 1938.**

The lyophile method of drying serum as reported by Flosdorf and Mudd appeared to the authors as a possible method for accomplishing the same purpose with blood typing serum. They have been able to dry serum by this method and redissolve the residue in less than the original volume. The resulting product was found to have a hemagglutinin titer greater than the unprocessed serum and to be suitable for use as test serum.

**TRICHINELLA SPIRALIS, Present Incidence of, as Determined by a Study of 1060 Unselected Autopsies in St. Louis Hospitals, Pote, T. E. Am. J. M. Sc. 197: 47, 1939.**

Muscle tissue from 1060 unselected autopsies were trichinous to the extent of 15.4 per cent. There had been no symptoms suggesting trichinella infestation.

Ninety-five per cent of the infestations were dead, calcified, and more or less disintegrated; 5 per cent were demonstrated to be alive by feeding the infested material to rats and recovering live trichinella larvae.

Very heavy infestations were to be observed where the individual gave a history of always having enjoyed good health. In one patient, dying at the age of 84, as the result of an accident, autopsy did not show that the infestation had contributed to the cause of death.

In 1500 hog carcasses examined for trichinella, 0.8 per cent were found to be infested, which gives rise to the question whether man is getting all of his trichinella infestation from pork. It may be concluded that man's infestation comes from pork derived from the small packing plants, which are not supervised by meat inspection services. It is to be noted that the infestations that sporadically occur are from pork from the establishments not under federal inspection.

It can be very definitely concluded that the *trichinella spiralis* infestation of man is not a serious lethal factor in Missouri, since in 163 instances of infestation it was not held to have been the cause of death or to have contributed to the cause of death.

# ENDOCARDITIS, Bacterial, Mechanism of Localization of Vegetations of, Allen, A C Arch Path 27 399, 1939

In from 50 to 75 per cent of cases bacterial endocarditis is superimposed on rheumatic endocarditis. Rheumatic endocarditis commonly produces a valvular fibroplastic deformity with stenosis. This lesion takes the form of a projecting shelf or barrier, usually at the line of closure, against which the blood stream strikes. By virtue of this obstruction to the systolic discharge (manifested by myocardial hypertrophy) the site of the deformity suffers a distinctly greater impact and contact than the normal valve leaflet, which "gives" or yields with the stream. This contact with the blood (and organisms in a bacteremia) is further enhanced by the diastolic backflow due to insufficiency—the usual concomitant of stenosis. The role of these dynamics in the localization of vegetations is suggested.

This same mechanism applies to congenital lesions including the "so called congenitally bicuspid aortic valve."

Attention is called to the fact that the outflow surface of all valves normal or deformed comes in contact with a much greater area of blood (and toxic agents) than the opposite surface. The significance of this in the localization of vegetations is stressed.

An explanation based on the same principles is offered for the rarity with which auricular fibrillation is complicated by bacterial endocarditis. The possibility of the influence of other auxiliary factors is not precluded.

It is pointed out that there is an increased tendency for acute rather than subacute endocarditis to occur on (a) valves not previously deformed and (b) valves of the right side of the heart. This fact is correlated with the principles of impact and contact.

## BLASTOMYCOSIS, Martin, D S, and Smith D T. Am Rev Tuberc 39 488, 1939

From a study of the recorded cases of blastomycosis and their own series of 13 cases, the authors have drawn the following conclusions:

American blastomycosis (Gilchrist's disease) is a distinct clinical entity caused by a specific etiologic agent, *Blastomyces dermatitidis*.

Two clinical types of infection are recognized: (a) cutaneous blastomycosis, which proceeds as a chronic or subacute ulcerating process and which usually responds to treatment with iodides or radiation, and (b) systemic blastomycosis, a highly fatal disease, characterized by pulmonary infection and widespread distribution of lesions in the subcutaneous tissues, bones, joints, central nervous system, and internal organs.

The same fungus, *Blastomyces dermatitidis*, causes both types of infection.

The disease is far more common in males, and the natural habitat of the fungus is evidently in nature, although proof of its existence in the environment has not been found.

Antibodies can be found in the sera of patients who are heavily infected, they persist until death unless the infectious process is overcome or greatly reduced.

Some patients develop a condition of hypersensitiveness to the infecting fungus, and this allergic state diminishes in the terminal stages of the disease. The degree of hypersensitiveness can be estimated by skin tests and can be materially reduced by the repeated injections of minute doses of a heat killed vaccine.

Potassium iodide is curative in some cases, but it is a dangerous drug to administer to patients who are allergic to the fungus.

In systemic blastomycosis iodide therapy should be started only after the state of hypersensitiveness has been excluded by skin test or artificially reduced by therapeutic injections of vaccine.

## TUBERCULOSIS, Culture Methods in the Diagnosis of, Whitehead, H G. Am Rev Tuberc 39 540, 1939

A total of 215 specimens of various types were examined for tubercle bacilli by direct and concentrated smears, cultures on two tubes each of four types of media and animal inoculation. Concentration did not increase the number of positive specimens, but satisfactorily prepared the material for culture and injection into a guinea pig. Culture and animal inoculation increased the number found on smear.

Petragnani's medium showed more efficiency in promoting growth of tubercle bacilli and more resistance to contamination than did the media recommended by Loewenstein, Corper, or Petroff for isolation from contaminated materials.

A series of 190 specimens without animal controls showed a similar superiority of Petragnani's medium.

Greater efficiency of the culture method is obtained by using several different types of media in routine laboratory search for the tubercle bacillus. Available data do not warrant the substitution of the culture method for the animal method, but indicate that both should be used for the maximum reliability in laboratory diagnosis of tuberculosis.

#### **ENDOMETRIAL BIOPSY, Clinical Interpretation of, Katz, J., and Parker, E. *Endocrinology* 24: 447, 1939.**

Three hundred endometrial biopsies from completely controlled patients in whom ovarian dysfunction was suspected have been studied and classified. Abnormalities which occurred in every stage of development have been described and interpreted in terms of disturbance of the functional level of the ovarian hormones.

Abnormalities of the endometrium reflect either failure of orderly elaboration or a disturbance in the normal balance of the two ovarian hormones at various times in the menstrual cycle. Pseudomenstruation, which is often considered to be synonymous with anovulatory menstruation, is not uncommon, occurring in 40 cases of this series.

Subnuclear vacuolization, which has been described as diagnostic of the occurrence of ovulation, is exceedingly variable in time of occurrence and may be superimposed upon an abnormal follicular type of endometrium. Secretory differentiation may be instituted in an endometrium showing evidence of either deficient or excessive follicular proliferation. The corpus luteum does not function in an "all-or-none" fashion, but it may elaborate an excess of one hormone (estrone) or a deficiency of one or both hormones.

There are no specific endometrial patterns for the gynecologic symptoms, with the possible exception of amenorrhea, in which a follicular endometrium is usually encountered. Pathologic bleeding may occur from any type of endometrium described. These same types occur in patients in whom there is no tendency to bleed. Menopause has no specific endometrial pattern. The endometrium merely reflects the degree of ovarian failure.

#### **PNEUMONIA, Giant Cell of Infancy as a Manifestation of Vitamin A Deficiency, Chown, B. *Am. J. Dis. Child.* 57: 489, 1939.**

The hypothesis that the giant cells of giant-cell pneumonia represent metaplasia of alveolar epithelium due to vitamin A deficiency cannot be proved by argument. Proof or disproof must await experiment. So, too, only experiment can answer the questions of whether infection can cause either local or general depletion of vitamin A, and whether such depletion can be prevented by giving the vitamin during the course of infection. It is an interesting problem.

Five cases of giant-cell pneumonia are reported, and the possibility that the giant-cell formation is the result of vitamin A deficiency is discussed, though no conclusion is reached. The effect of infection on vitamin A depletion is also considered.

#### **TULAREMIA, Diagnosis of, Friedenwald, W. F., and Hunt, G. A. *Am. J. M. Sc.* 197: 493, 1939.**

The results of diagnostic tests in 50 cases of tularemia are presented.

The blood agglutination is the most constant and reliable diagnostic method after the first week of the disease.

The antigen skin test is positive during the first week of the disease and appears to be highly specific and reliable.

The antiserum skin tests, both goat and horse, have been of value in only a small percentage of cases, because of nonspecific reactions of control sera. It is always necessary to use a control serum in this test.

The opsonocytaphag reaction tends to parallel the blood agglutination in time of appearance and is valuable in differentiating Brucellosis from tularemia, when cross agglutination occurs.

# DERMATOSIS, Precancerous and Epithelioma in Situ, Montgomery, H. Arch Dermat & Syph 39 389, 1939

In the group of precancerous dermatoses are included true Bowen's disease, senile keratoses, keratoses resulting from arsenic, tar, or radiation and leucoplakia of the mucous membranes. In 20 per cent or more of the patients epithelioma of varying degrees of malignancy eventually will develop, usually starting as squamous cell epithelioma in situ. The conditions are all interrelated by their common histologic picture and hence, a possible common carcinogenic factor. They are not to be confused with the relatively rare epithelioma in lesions of lupus vulgaris or other benign dermatoses such as psoriasis and lichen planus. Precancerous dermatoses also must be distinguished from various conditions which result in the histologic picture of pseudoepitheliomatous hyperplasia. The incidence of epithelioma associated with lupus erythematosus or with scars from burns or other causes will vary up to 10 per cent in cases of such involvement but the senile cutaneous changes, the age of the patient, the situation of the lesions, including the factors of repeated trauma and irritation, and the previous treatment employed must be considered. In many cases of precancerous dermatosis, histologic transitions between a benign and a malignant process may be seen in the same section.

The peculiar or distinctive and predominant histologic feature of epithelioma in situ with the phenomena of Bowen's disease, together with their relatively long duration as such, without clinical or histologic evidence of malignant invasion justify the grouping of true Bowen's disease, senile keratosis, keratosis resulting from arsenic, tar, and radiation and in most cases leucoplakia of the mucous membranes under the title "precancerous dermatoses." If the term "precancerous dermatosis" is used in the limited sense defined it has a definite meaning both clinically and pathologically and is of value from the standpoint of prognosis and treatment until more is known about the cause of cancer. The term "precancerous dermatosis," however, should not be used loosely when the pathologist is in doubt regarding the clinical or histologic picture despite the fact that at present there is no general agreement regarding the distinction between cancer and precancerosis of the skin.

# PAGET'S DISEASE, Extramammary and Intraepidermal Carcinoma Pinkus, H., and Gould, S. E. Arch Dermat & Syph 39 479 1939

A discussion is given of the nature and pathogenesis of the disease reported in the literature as extramammary Paget's disease.

Three cases are presented which offer, respectively, clinical, histologic and clinical and histologic features of Paget's disease.

Criteria are discussed by which Paget's disease can be differentiated from similar diseases (Bowen's disease, erythroplakia, and superficial epidermoid carcinoma).

In accordance with the views of previous authors, Paget's disease of the nipple is defined as the expression of a more or less well balanced symbiosis of the epidermis and an epidermotropic strain of cancer cells of mammary origin.

It is proposed to designate the interesting and unique biologic phenomenon of symbiosis of epidermis and "Paget cell" as the "Paget phenomenon."

On the basis of this biologic conception it appears justifiable to include as instances of Paget's dermatosis all those cases in which the Paget phenomenon is present, Paget's disease of the nipple being only its most frequent and best known example.

Extramammary Paget's dermatosis is due to the intraepidermal spread (a) of carcinoma of the apocrine glands (and perhaps of other cutaneous glands), (b) of carcinoma of mucous membranes bordering on the skin, and (c) possibly of melanoblastoma.



**LAUGHLIN TEST**, Inadequacy of the, and the Ide Test in the Diagnosis of Syphilis, Flood, J. M., and Mayer, V. Arch. Dermat. & Syph. 39: 510, 1939.

A comparative study of the Laughlen and the Ide tests with the Kline, Kahn, and Kolmer tests is presented.

Tests were performed according to directions supplied by the distributors of the antigens.

The Laughlen and the Ide tests are less specific than the three standard tests used.

The Laughlen and the Ide tests are inadequate for the serodiagnosis of syphilis.

The Ide test is satisfactory for the examination of spinal fluid, but the Laughlen test grossly fails when the spinal fluid is not concentrated.

**BLOOD, Preserved, Citrated in "Banks" in Relation to Transfusion in the Treatment of Disease, With Special Reference to the Immunologic Aspects**, Kolmer, J. A. Am. J. Med. Sc. 197: 443, 1939.

While sodium citrate is slightly anticomplementary, the complement of citrated (0.35 per cent) human blood, kept at 4° to 6° C., was well preserved for periods of from fourteen to twenty-one days. The same has been found true of undiluted citrated guinea pig complement, kept at 2° to 4° C.

The bacterial activity of normal citrated human blood, kept at 4° to 6° C. for *Staph. aureus*, beta hemolytic streptococcus, and *B. coli*, decreased after seven to twenty-one days' preservation.

The phagocytic activity of the neutrophils of preserved citrated human blood, kept at 4° to 6° C. for *Staph. aureus*, beta hemolytic streptococcus, and *B. coli* was definitely reduced within seventy-two hours after collection of blood, becoming markedly so on or about the fifth day, followed by almost total absence of phagocytic activity on and after the seventh day of preservation. This may have been due to deterioration of normal opsonins but was mostly due to autolytic and degenerative changes in the leucocytes.

The erythrocytes of preserved citrated human blood, kept at 4° to 6° C., showed evidences of swelling and dehemoglobinization as early as forty-eight hours after collection, with progressive degenerative changes up to fourteen days, when at least 30 per cent were shadows, swollen and fragile.

The neutrophils of preserved citrated human blood, kept at 4° to 6° C., showed evidences of disintegration with reduction in numbers as early as twenty-four hours after collection, with progressive disintegrative and autolytic changes up to fourteen days (longer intervals of preservation not being employed).

The platelets of preserved citrated human blood, kept at 4° to 6° C., showed distinct clumping immediately and twenty-four hours after collection, with evidences of deterioration in the latter. At the end of forty-eight hours they became scarce, and after five days only blue chromatin masses remained.

For reasons discussed it is believed that citrated human blood, preserved at 4° to 6° C., may be useful in the treatment of acute hemorrhage and shock for the purpose of restoration of volume, but is inadvisable for the treatment of the anemias, the blood dyscrasias or infections, and certainly should not be employed for these purposes after two to three days' preservation.

**ANEMIA, Sick Cell, The Erythrocyte in**, Diggs, L. W., and Bibb, J. J. A. M. A. 112: 695, 1939.

Little information is to be obtained from the literature concerning the sedimentation velocity of erythrocytes in sickle-cell anemia. The sedimentation rate was determined for 23 patients in our series. The total number of determinations was 49. Oxalated blood and 5 c.c. Cutler tubes were used; the readings were made at five-minute intervals for one

hour In four instances the sedimentation rate was 5 mm or less in one hour, and in 14 instances the rate was less than 10 mm in one hour Repeated examinations of the sedimentation rate for the same person at different times revealed variable rates, although the degree of anemia did not significantly change Increased rates could usually be explained by some demonstrable abnormality, such as active tuberculosis, salpingitis, or badly infected ulcers of the legs, but in some cases there was no obvious cause for the increased rate The fact that the sedimentation rate may be normal in spite of severe anemia indicates that the increased rate in these cases is due to some complication whether it is clinically demonstrable or not Since the sedimentation rate may be normal in the presence of mixed anemia, it is obvious that correction factors based on the degree of anemia are not applicable in this disease

**TUBERCULOPROTEIN, Specific Effects of, Corper, H J, and Cobb, M L J A M A 112 403, 1939**

Specific immunity against infections with virulent human tubercle bacilli could not be produced by Seitz filtrates from cultures of virulent human tubercle bacilli, by highly concentrated (ultradialysis) filtrates, or by the precipitated tuberculoprotein or alum treated filtrates

The highly concentrated filtrates, or precipitated tuberculoprotein, possess no appreciable primary toxicity for normal animals

A primary intravenous injection of large amounts of normal Seitz filtrates, highly concentrated (by ultradialysis) filtrates or precipitated tuberculoprotein from these filtrates does not sensitize to a second intracutaneous injection with a fairly large test dose (0.1 mg) of either filtrate or tuberculoprotein A reaction to tuberculin can be obtained, however, in a tuberculous guinea pig with as little as 0.000005 mg of the same material

A primary intravenous injection of about 2 mg of tuberculoprotein in any of the foregoing forms sensitizes guinea pigs to a lethal intravenous provocative dose (anaphylactically) of as little as about 1 mg of the tuberculoprotein in these forms This confirms the studies of Zinsser, who worked only with large amounts of bacillary extracts and showed a striking difference between cutaneous and anaphylactic reactions

To produce cutaneous hypersensitiveness to tuberculoprotein requires a small amount of avirulent human tubercle bacilli (a tubercle forming dose), a large amount of heat killed tubercle bacilli (around 100 mg), and very small amounts of virulent tubercle bacilli With the latter, the factors of multiplication and tuberculous involvement complicate the picture

There is a striking quantitative difference between the specific immune and the concomitant allergic or anaphylactic features of tuberculosis which will eventually have to be given independent consideration in the complete evaluation of tuberculosis in man, since one is protective and the other shows a peculiar type of still unsolved intoxication

**BONE MARROW Sternal Marrow of Children in Normal and Pathologic States, Vogel P, and Bassen, F A Am J Dis Child 57 245, 1939**

The following method was used for the study of sternal marrow

The skin over the upper portion of the sternum was prepared with iodine and alcohol The site usually selected for puncture was at the level of the upper border of the third rib in the midline of the body of the sternum In infants and in young children, however, the manubrium was found to be preferable for aspiration, the reason being that this portion of the sternum is thicker and, therefore, safer for penetration, at the same time tending to fix the needle more firmly No anesthesia was necessary The sternal plate in children is so thin that pain is only momentary A  $\frac{1}{2}$  inch (1.27 cm), 18 gauge lumbar puncture needle was inserted into the marrow cavity at an angle of about 45 degrees, with the point directed cephalically For children under 1 year of age a 20 gauge needle of  $\frac{1}{4}$  inch (0.64 cm) length is preferable



## ITEMS

---

### Institute for the Consideration of Blood and Blood-Forming Organs

The University of Wisconsin Medical School will conduct an Institute for the Consideration of the Blood and Blood Forming Organs, September 4-6, 1939. The program will include papers and round table discussions by European and American workers in the field of hematology. In addition to the discussions, the following formal papers will be presented:

- Dr. L. J. Witts, Oxford, England, Anemias Due to Iron Deficiency  
Dr. Cecil J. Watson, Minneapolis, The Porphyrins and Diseases of the Blood  
Dr. Cornelius P. Rhoads, New York, Aplastic Anemia  
Dr. E. Meulengracht, Copenhagen, Denmark, Some Etiological Factors in Pernicious Anemia and Related Mierocytic Anemias  
Dr. Harry Eagle, Baltimore, The Coagulation of Blood  
Dr. George R. Minot, Boston, Anemias of Nutritional Deficiency  
Dr. Russell L. Haden, Cleveland, The Nature of the Hemolytic Anemias  
Dr. Jacob Furth, New York, Experimental Leucemia  
Dr. Claude E. Forkner, New York, Monocytic Leucemia and Aleucocytic Leucemia  
Dr. Edward B. Krumbhaar, Philadelphia, Hodgkin's Disease  
Dr. Louis K. Diamond, Boston, The Erythroblastic Anemias  
Dr. Edwin E. Osgood, Portland, Marrow Cultures  
Dr. Charles A. Doan, Columbus, The Reticulo Endothelial System  
Prof. Hal Downey, Minneapolis, Infectious Mononucleosis  
Dr. Paul Reznikoff, New York, Polycythemia

Physicians and others who are interested are cordially invited. A detailed program may be obtained by addressing Dr. Ovid O. Meyer, Chairman of Program Committee, University of Wisconsin Medical School, Madison, Wis.

---

### Biological Photographic Association Convention

The Ninth Annual Convention of the Biological Photographic Association will be held September 14 to 16, at the Mellon Institute for Industrial Research, Pittsburgh, Pa. The program will be of interest to scientific photographers, scientists who use photography as an aid in their work, teachers in the biological fields, technical experts, and serious amateurs. It will include discussions of motion picture and still photography, photomicrography, color and monochrome films, processing, etc., all in the field of scientific illustrating. Modern equipment will be shown in the technical exhibit, and the Print Salon will display the work of many of the leading biological photographers here and abroad.

The Biological Photographic Association was founded nine years ago because of the growing need for expert illustrative material for scientific research and teaching. Many workers were solving their problems in their own way. But obviously they were wasting their time and effort in individually repeating experiments that had been worked out elsewhere. The B.P.A. was formed to act as a clearinghouse for new ideas, to pool experiences, to record standard procedures, and to disseminate information. Its aims were scientific, and all services have been volunteered by officers and members on a nonprofit basis.

The B.P.A. Journal is published quarterly, constituting a volume of about 250 pages, and is furnished free to members. Membership privileges include an authoritative question and answer service; also the right to borrow loan albums and exhibits of scientific prints for study and display.

Further information about the Association and the Convention may be obtained by writing the Secretary of the Biological Photographic Association, University Office, Magee Hospital, Pittsburgh, Pa.

## CORRESPONDENCE

---

Boise, Idaho,  
May 20, 1939.

The Editor,  
JOURNAL OF LABORATORY AND CLINICAL MEDICINE.

Dear Sir:

Page 891 of your May, 1939, publication contains an abstract of an article by A. E. Platt entitled "Salt Optima in Agglutination," with special reference to *Brucella abortus*, in which the author states that the antigen-antibody optimal ratios remained constant for all salt concentrations between 2M/7 and M/56. In this connection, it may be of interest to you to know that in Bang's disease agglutination tests performed in this laboratory a 50M/58.5 concentration of sodium chloride was found to give the most complete agglutination, that is to say, no hazy reactions. When this concentration of salt is combined with 0.02 c.c. of a 1 per cent solution of brilliant green and 0.02 c.c. of a 1 per cent solution of gentian violet per 100 c.c. of the salt solution, a very satisfactory antigen for Bang's disease is obtained.

HARRY H. UTEB  
State Department of Agriculture

# *The Journal of Laboratory and Clinical Medicine*

VOL 24

AUGUST, 1939

No 11

## *CLINICAL AND EXPERIMENTAL*

### THE EFFECT OF VITAMIN C ON LEAD POISONING

HARRY N HOLMES, PH D, KATHRYN CAMPBELL OBERLIN, OHIO, AND  
EDWARD J AMBERG, M D TOLEDO OHIO

IN a large industrial plant where the lead hazard was great due to fine metallic lead dust from filing down lead alloy seams lead fumes from melting pots, and lead contacts from putty spray guns there was an exceptional opportunity to diagnose and treat lead absorption and poisoning

Four hundred men exposed to lead during their daily tenure at the plant were examined physically, and by laboratory methods The latter consisted of monthly checks on the basophilic aggregation (B A), of the blood smear, according to McCord,<sup>1</sup> and on the degree of stippling, using Wright's stain Records of these monthly tests were kept throughout the period of observation from April, 1937, until April 1, 1938 During the last three months of this period weekly tests were recorded on a cooperative group of 34, all of whom had symptoms and showed signs of lead poisoning In addition to the above mentioned tests differentials were recorded on the Wright's smear, using the classification of Failey and others,<sup>2</sup> as used by Steinberg\* Urinalyses were not done due to technical difficulties at the factory

During the period from April 1, 1937, to November 15, 1937, the gluconate, lactate, and diphosphate salts of calcium were given in the amount daily of from 80 to 100 grains to those who showed lead absorption by physical or laboratory signs In addition, vitamin D (940 units) and vitamin A (9400 units) were given daily, using an accredited fish liver oil

The criterion used was a basophilic aggregation percentage of 15 or greater, abnormal stippling, or by request of the ill individual Biweekly intravenous injections of 10 cc of 10 per cent calcium gluconate, together with daily oral therapy with calcium salts and vitamin D,<sup>1</sup> were given to

\*Dr Bernhard Steinberg Director of Laboratories Toledo Hospital  
Received for publication November 14 1938

those whose basophilic aggregation registered higher (3.0 plus). Deleaching with halides of potassium and ammonia were not done.

The period of intimate observation of the group of 34 lead poison patients upon whom we base this report began in November, 1937. It was then noted that, in spite of the intensive treatment with calcium and vitamin D, and the prophylactic measures taken by the plant management to reduce the lead hazard, many of the men failed by physical and laboratory standards to recuperate. In some, while there was a return to normal in percentage of basophilic aggregation and degree of stippling, there remained the complaint of ill-health, or abnormal cell morphology, or both.

Most frequent complaints were fatigue, anorexia, sleeplessness, or restless sleep; pains in the legs (gastrocnemius muscles) or in the forearms near the elbow; tenderness on deep pressure over the same areas, and irritability. Less frequently, complaint was made of trembling, constipation, sexual impotence, and joint pain. Observations on these patients consisted mainly of pyorrhea, spongy gums, and poor dentition in 75 per cent; tremor of varying degree, involving fingers and hand, usually bilaterally, and pallor of varying degree in 50 per cent; weakened extensibility at the wrist in about 10 per cent. One of the most striking signs was the surliness encountered in the majority of these subjects.

*Ascorbic Acid Treatment.*—One of us (E. J. A.) made the observation that the symptoms and signs exhibited by these men were similar to those found in subclinical scurvy. Ascorbic acid oral therapy was instituted in a group of 34 patients with chronic lead poisoning according to the following plan: Of 34 workmen (chronic patients) selected for this novel treatment, 17 were given ascorbic acid alone (at least two months after discontinuing the calcium salt injections), while an equal number continued the calcium therapy and at the same time took tablets of ascorbic acid.

*Ascorbic Acid Without Calcium.*—In general, the group of 17 patients made a marked gain in vigor, cheerfulness, color of skin, and blood picture. A week, or less than a week, after beginning the treatment of 100 mg. of ascorbic acid daily, most of the men enjoyed normal sleep, lost the irritability and nervousness so common with high calcium treatment of lead poisoning, enjoyed their food more, and no longer had tremors (if observed before). Several cases of leucopenia (probably due to a prolonged calcium therapy somewhat earlier) were cured by the ascorbic acid treatment.

*Ascorbic Acid With Calcium.*—The gain was less marked and rather irregular in the 17 patients given ascorbic acid with calcium. There was no gain in the number of mature neutrophils in the blood—in strange contrast with the rapid gain when ascorbic acid alone was given.

In several instances alcohol nullified the good effects of calcium as well as of ascorbic acid, a complication that workmen exposed to lead hazards would do well to avoid.

#### CASE REPORTS

The detailed blood picture and the clinical observations for 3 workmen who received benefit from vitamin C in chronic lead absorption are outlined: The blood count was for 200 cells.

## WORKMAN 1

## CALCIUM GLUCONATE ALONE LATE ASCORBIC ACID ALONE

DATE	B A * %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MAJORITY NEUTRO PHILES %	STABS %	JUV NILES %	MYELO CYTES %	LYMPHO CYTES %	EOSINO PHILES %
1937 June Late June	6	Strong							
Began biweekly intravenous injections of 10 cc 10 per cent calcium gluconate plus 0 grains calcium salts orally									
July Aug 1	43 33	Strong Strong							
Discontinued calcium therapy Plant closed Aug 10 to Sept 9 Lead hazard greatly reduced during September									
Oct Nov	30 22	Positive Mild							
Feb Feb 9	14	Negative	Anisocytosis	24	2	0	2	68	4
Begin daily doses of 100 mg ascorbic acid									
Feb 21	18	Strong		36	4	8	0	50	2
Feb 28	15	Strong	Anisocytosis marked	40	0	-	0	55	2
Mar 9	12	Mild	Normal	46	9		0	48	1
Mar 16	10		Normal	66	2	1	0	18	6
Treatment discontinued									

\*Basophilic aggregation

Monocytes none from Feb 9 to March 16

Basophiles 0 0 1% 0 4% from Feb 9 to March 16

Clinically Workman 1 on February 9 showed definite symptoms of chronic lead poisoning with marked tremor, nervousness, irritability, sleeplessness. He was underweight, tired easily, and had a very sallow complexion. His blood picture was discouraging.

By March 16, after five weeks of ascorbic acid treatment the tremor disappeared and his complexion greatly improved. The man was very cheerful and no longer tired easily. The blood picture also greatly improved.

## WORKMAN 2

## CALCIUM SALT THERAPY LATE ASCORBIC ACID ALONE

DATE	B A * %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MAJORITY NEUTRO PHILES %	STABS %	JUV NILES %	MYELO CYTES %	LYMPHO CYTES %	EOSINO PHILES %
1938 Feb 9	34	Positive	Anisocytosis moderate	8	0	6	2	18 (?)	0
Feb 10		Begin biweekly intravenous injections of calcium gluconate Calcium lactate and Natol orally daily							
Feb 21	14	Negative	Achromia, anisocytosis slight	34	2	4	0	44	0
Feb 24	09	Negative	Anisocytosis moderate	49	2	6	0	9	3
Mar 3	31	Strong	Anisocytosis, poly chromasia, achromia moderate	40	2	8	0	44	4
Mar 7	12	Negative	Anisocytosis moderate	10	2	4	4	48 (?)	0
Mar 7	Begin daily doses of 100 mg ascorbic acid Calcium discontinued								
Mar 14	13	Negative	Normal	62	2	0	0	34	1

\*Basophiles 0 6% 1% 0 1% 0 for the six dates

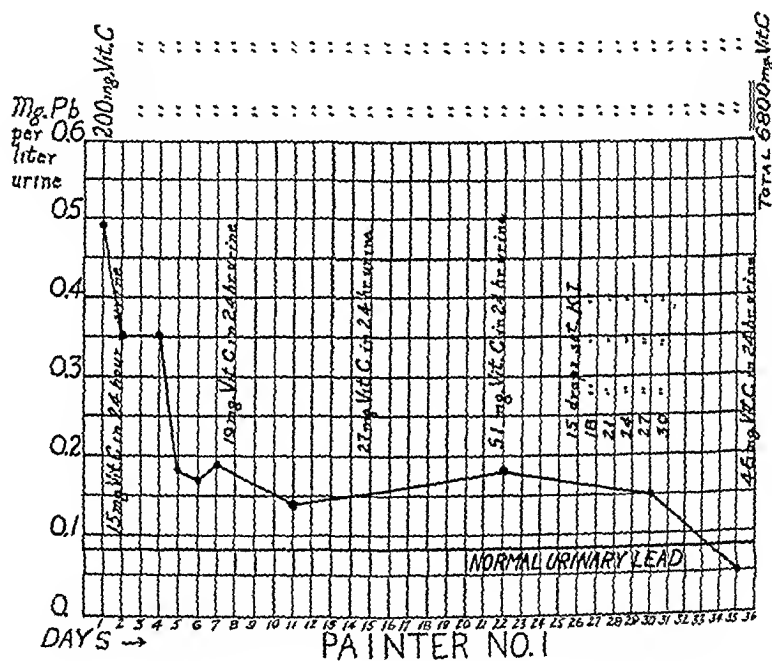
Monocytes 6% 10% 0 2% 0 1% for the six dates



Clinically Workman 2 showed symptoms of chronic lead poisoning, with moderate tremor, nervousness, irritability, sleeplessness, and pallor. He tired easily. One week after beginning the ascorbic acid treatment, tremor and pallor were gone. He slept well, did not tire, and was extremely cheerful and talkative.

Workman 3 displayed clinically an acute case of lead poisoning in June, 1937, with a basophilic aggregation of 14, strong stippling, etc. Calcium therapy from June to February, 1938, lowered his basophilic aggregation satisfactorily, but three weeks after discontinuing this treatment, it increased considerably with return of the symptoms of acute lead poisoning.

He was then given 100 mg. of ascorbic acid daily for sixteen days and his basophilic aggregation was again lowered to normal (as with calcium therapy), with no clinical symptoms of lead poisoning. The morphology of the red blood cells became nearly normal. The man looked well and felt fine.



**House Painters.**—The research was transferred to Oberlin to make a study of ordinary house painters. Here it was possible to make analytical determinations of lead and ascorbic acid in the urine. The dithizone method for urinary lead, as described by Ross and Lucas,<sup>2</sup> and improved, especially in the use of a calculation curve, by Miller<sup>3</sup> and his associates, was used.

For the determination of ascorbic acid in urine the titration against the dye, 2,6-dichlorophenolindophenol, as described by Harris and Ray,<sup>4</sup> or Hawk,<sup>5</sup> was used. We found it necessary to devise improved methods of collecting twenty-four-hour specimens of urine in order to prevent the usual considerable losses of vitamin C (ascorbic acid) by oxidation. The details are given in another paper. Tests with a number of healthy people convinced us that the common estimate of an average daily urinary excretion of 15 mg. of vitamin C is too low, due to this oxidation loss, and that 25 mg. to 35 mg. is the probable average for healthy persons.

Of 14 painters observed clinically and by differential blood count, 2 were rated by clinical examination as chronic lead absorption patients, and the blood pictures added 5 more. Three of the 7 patients refused to cooperate further; hence we selected only 3 for study and treatment. Seven of the 14 picked up at random showed enough absorption to call for treatment or greater precautions.

## CASE REPORTS

Case histories of 3 painters are here given. The final brief deleading was done by Dr A C Siddall, who gave somewhat more potassium iodide than usual. In order to demonstrate more convincingly the value of a diet rich in vitamin C for men exposed to lead hazards, we determined the urinary lead and vitamin C of 2 more painters and gave them a brief deleading treatment. Furthermore, we determined the urinary vitamin C and lead of another painter who for many years had stressed fruits, vegetables, and milk in his diet.

Clinically the first painter and sprayer, who exhibited symptoms of chronic lead poisoning (physician's report) responded quickly to ascorbic acid treatment. After four days, he felt lazy and sleepy but not tired. Then he quickly changed to "feeling fine," ready for work, and fresh at the end of a hard day's work. At the same time there was a decided improvement in his blood picture. His urinary lead quickly fell to nearly normal, and his ascorbic acid excretion rose from 15 mg per twenty-four hours to the very high value of 51 mg.

## PAINTER 1

## ASCORBIC ACID TREATMENT FOLLOWED BY DELEADING

DATE	B. A. %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTRO PHILES %	STABES %	JUVE NILES %	MELO CYTES %	LYMPHO CYTES %	EOSINO PHILES %
1938									
May 11	0.9	7,500	Anisocytosis and slight poly chromasia	32	9	5	0	43	5
July 7	1.0	6,000	Anisocytosis and slight poly chromasia	30	10	6	0	45	6
July 7		Began	200 mg ascorbic acid daily						
July 19	2.2	8,000	Anisocytosis moderate	50	10	4	0	31	3
Aug 1		Continued	ascorbic acid and began deleading with potassium iodide						
Aug 7		End of	deleading						
Aug 9	1.6	Negative	Normal	62	2	1	0	30	2
Aug 10		End of	ascorbic acid treatment						

Basophiles 2% May 11, 2% July 7, 2% July 19, 1% Aug 9

Monocytes, 1% May 11, 1% July 7, 0 July 19, 2% Aug 9

Clinically Painter 2 was rated by one of us (E J A) as showing symptoms of chronic lead poisoning, even to a slight lead line on the gums. His teeth were bad and he tired quickly.

Two days of ascorbic acid treatment made him lazy and sleepy. After three weeks of ascorbic acid, he felt fine and energetic at the end of a hard day's work. His family noted the change from irritability to cheerfulness.

Urinary lead excretion fell from 0.4 mg per liter to about 0.1 mg. Ascorbic acid in twenty-four hours' urine rose from 12 mg to 86 mg.

Clinically Painter 3 seemed to one of us (E J A) to be in good health, showing no signs of lead poisoning and having no complaints. During the period from May 9 to May 23 his blood picture was not reassuring and plans were made for ascorbic acid

## PAINTER 2

## ASCORBIC ACID TREATMENT CONTINUED THROUGH DELEADING

DATE	B. A. %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTRO- PHILES %	STABS %	JUVE- NILES %	MYELO- CYTES %	LYMPHO- CYTES %	EOSINO- PHILES %
1938									
May 5	2.1	Negative	Anisocytosis slight	35	4	8	0	52 (†)	1
July 7	2.5	Negative	Anisocytosis moderate	28	6	10	0	54	2
July 7		Began 200 mg. ascorbic acid daily							
July 14	2.1	3,500	Anisocytosis moderate, poikilo- cytosis slight, poly- chromasia slight	40	15	4	1	36	2
July 28	1.4	Negative	Normal	64	2	1	0	28	3
July 29		Discontinued ascorbic acid. Deleading with potassium iodide began and continued three days until an attack of colic							
Aug. 9	2.3	3,000	Anisocytosis moderate, poikilo- cytosis slight, poly- chromasia slight	32	7	1	0	54	4

Basophiles, 2% Aug. 9. None on other dates.

Monocytes, 0 May 5, 0 July 7, 2% July 14, 2% July 28, 0 Aug. 9.

treatment. Urine examined for lead showed 0.5 mg. per liter (as compared to the average man's 0.085 mg.). Vitamin C excretion per twenty-four hours was very low, being only 10 mg.

Two weeks of ascorbic acid treatment decidedly improved his blood picture and astonishingly lowered the urinary lead to 0.18 mg. per liter. Ascorbic acid excretion rose to the level of 31 mg. daily.

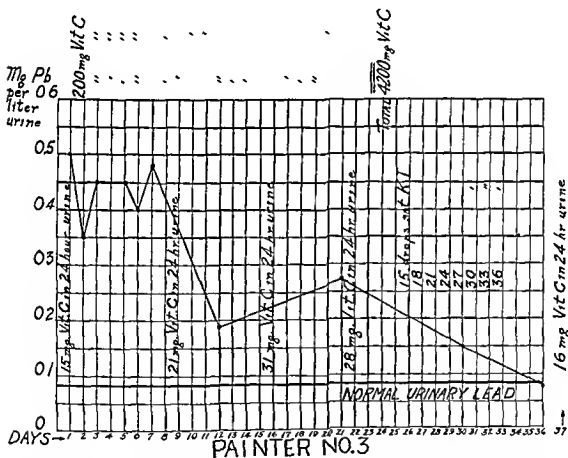
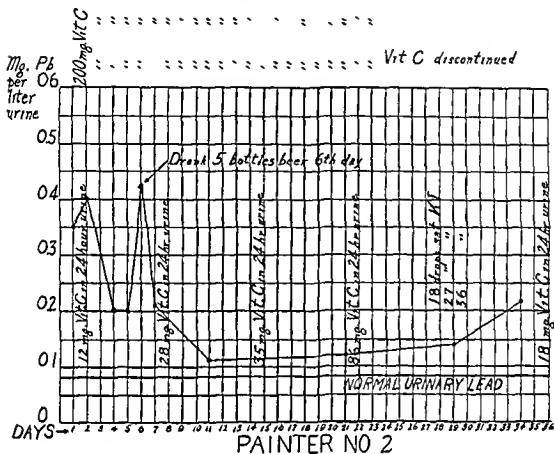
## PAINTER 3

## ASCORBIC ACID TREATMENT FOLLOWED BY DELEADING

DATE	B. A. %	STIPPLING	RED BLOOD CELL MORPHOLOGY	MATURE NEUTRO- PHILES %	STABS %	JUVE- NILES %	MYELO- CYTES %	LYMPHO- CYTES %	EOSINO- PHILES %
1938									
May 9	1.8	30,000	Anisocytosis and poly- chromasia moderate	44	13	2	1	26	10
May 23	3.2			34	24	2	0	31	7
July 7	2.0	15,000	Anisocytosis moderate, poikilo- cytosis slight	30	12	3	0	47	6
July 7		Began 200 mg. ascorbic acid daily							
July 21	1.6	Negative	Normal	58	2	2	0	34	3
July 29		Discontinued ascorbic acid. Began deleading with potassium iodide (15 drops saturated solution, increasing by 3 drops daily)							
Aug. 8	1.0	Negative	Normal	57	4	1	0	35	3

Basophiles, 2% May 9, 2% May 23, 2% July 7, 1% July 21.

Monocytes, 2% May 9. None on other dates.



Eight days of deleading (ascorbic acid discontinued) brought urinary lead to normal (0.085 mg per liter), but the ascorbic acid excretion unfortunately fell to 16 mg per twenty four hours. Clinically, the man seemed in good health throughout the deleading treatment.

**Painter 4**—Painter 4, a house painter for twenty five years, showed the effects of a diet unusually rich in the best vitamin C foods as well as in milk. His health (except for a temporary tooth infection) was good, his urinary lead was only 0.1 mg per liter, and his ascorbic excretion was 58 mg per twenty four hours, an exceptional figure.

Deleading with 80 grains ammonium chloride daily for six days was done (no milk in that period) and the urinary lead was cut to 0.04 mg per liter.

*Painter 5.*—The son of Painter 4, Painter 5 was brought up in the same dietary tradition as his father. He was in excellent health and painted for several years. He excreted 0.16 mg. lead per liter of urine. After six days of deleading with ammonium chloride (80 grains daily), his urinary lead excretion fell to 0.05 mg. He was comfortable during deleading. The usual vitamin C excretion had been 40 mg. per twenty-four hours urine.

*Painter 6.*—Painter 6 was a house painter who for fifteen years faithfully applied the dietary teachings of a religious organization and lived on a diet exceptionally rich in fruits and vegetables (many raw) and milk. He was in excellent health and possessed a good blood picture and a very good vitamin C excretion of 37 mg. per twenty-four hours. His lead excretion was 0.215 mg. per liter after four days of deleading with potassium iodide.

His wife on the same religious diet excreted 50 mg. of vitamin C per twenty-four hours, but in her case the effect of lead on vitamin C was not a factor. His pastor, a painter for thirty years, also living on a diet exceptionally rich in fruits, vegetables, and milk, enjoyed splendid health, and excreted only 0.13 mg. lead per liter in spite of his painting.

### DISCUSSION

Administration of 100 mg. of vitamin C daily to each of 34 workmen exposed to factory lead hazards (and diagnosed as suffering from lead absorption) in general decidedly improved their blood picture and their health. Symptoms characteristic of chronic lead absorption usually disappeared. It seems reasonable to suppose that toxic lead ions were removed to a considerable extent from the blood stream. Calcium gluconate or lactate does this by storing the lead in the bones, presumably as lead phosphate. Experience shows some drawbacks to long-continued high calcium medication; hence bone storage should be followed by cautious deleading, with a respite from lead absorption.

A more detailed study of 3 house painters, diagnosed as suffering from chronic lead absorption, strongly reinforced the conclusions drawn from the factory experiments. The observation that 200 mg. daily of vitamin C as a dietary supplement decidedly lowered the urinary excretion of lead is astonishing. With it goes another observation that men actually suffering from lead absorption excrete less vitamin C in the urine than does the average man.

It is difficult to escape the conclusion that toxic lead compounds react with vitamin C to form a poorly ionized salt (lead ascorbate?) or a complex salt which yields a very low concentration of simple lead ions. If solutions of lead acetate and vitamin C are mixed in a test tube, no precipitate is observed; hence the product is soluble, not to be precipitated in the bones. Cautious addition of sodium hydroxide fails to precipitate lead hydroxide or oxide, normally termed insoluble.

This view of removal of toxic lead ions by reaction with ascorbic acid to form soluble, but poorly ionized, salts or complexes is strengthened by the observation of Greenwald<sup>6</sup> on the remarkable influence of ascorbic acid on the dissociation (and solubility products) of calcium phosphate and certain other calcium salts. He is of the opinion also that calcium gluconate, lactate, and citrate form complexes with calcium ions. Hastings and McLean<sup>7</sup> offer a parallel in the fact that citrates lower the concentration of free calcium ions through formation of a soluble complex.

In an excess of enthusiasm over the good diet, one of our painters drank a pint of milk for five days before starting the ascorbic acid treatment.

His vitamin C excretion, previously only 10 to 13 mg, quickly rose to 25 mg and, after he discontinued his daily pint of milk, quickly fell to 15 mg. A similar observation was made with another painter. Evidently sufficient dietary calcium spares vitamin C in the body of a man suffering from lead absorption, probably by forcing some of the lead into the bones as lead phosphate.

A natural fear arises that if ascorbic acid therapy reduces urinary excretion of lead this lead must be stored somewhere in the body, to be released later in toxic form in the blood stream as dietary or other conditions change. This was not true of four or five local house painters who enjoyed several, even many, years of excellent health while painting, evidently because of a diet that we know to be extremely rich in vitamin C. At any rate, we found that they excreted more of this vitamin than does the average healthy individual.

Judging from the statement by Sollman on chelating with potassium iodide, it seems a fair assumption that the compound formed by ascorbic acid and toxic lead ions is preferentially absorbed by the liver and, with the aid of the bile, excreted in the feces. We hope that others interested in this research will determine this by quantitative analysis. He<sup>8</sup> writes "Renal excretion of lead depends directly upon the lead compounds circulating at the time in true solution in the blood. The increased excretion (of lead) through use of potassium iodide has been noted by previous workers, although others have failed to confirm it, chiefly because they confined themselves to the urine while the excretion is mainly in the faeces. The iodide causes the reappearance of lead in the faeces from which it had been absent."

*Note*—An extra 50 mg more or less of vitamin C is furnished by approximately

4 oz fresh orange juice	1 oz raw green pepper (salad)
4 oz grapefruit	8 oz tomatoes whole or juice
4 oz raw cabbage	8 oz cantaloupe
2 oz raw spinach (salad)	4 oz raw turnips
4 oz spinach, cooked in least possible water	

In general, fresh fruits and vegetables (and canned tomatoes) are very good sources of vitamin C, but the average workman will find the above list definite and practical. On a limited income even raw cabbage and turnips with canned tomatoes are possible as sources of 50 mg or 100 mg vitamin C daily.

#### REFERENCES

- McCord, C. P., and Johnson, J. The Basophilic Aggregation Test. *Am J Pub Health* 25: 1089, 1935.
- Ross, J. R., and Lucas, C. G. A New Method for the Determination of Minute Amounts of Lead in Urine. *J Biol Chem* 3: 290, 1935.
- Winter, O. B., Robinson, H. M., Lamb, F. W., and Miller, E. J. The Determination of Lead, *Indust & Engin Chem Analyt Ed* 7: 265, 1935.
- Harris, L. J., and Ray, S. N. Diagnosis of Vitamin C in Subnutrition by Urine Analysis, *Lancet* 1: 71, 1935.
- Hawk, P. B., and Bergheim, O. *Practical Physiological Chemistry*, ed 11, Philadelphia 1937, P. Blakiston's Son & Co., p 733.
- Greenwald, I. The Dissociation of Some Calcium Salts, *J Biol Chem* 124: 437, 1938.
- McLean, F. C., and Hastings, A. B. A Biological Method for the Estimation of Calcium Ion, *J Biol Chem* 107: 351, 1934.
- Sollman, T. A Manual of Pharmacology, ed 3, Philadelphia, 1928, W. B. Saunders Co., p 1049.
- Farley, D. L., St. Clair, H., and Reisinger, J. A. *Am J M Sc* 180: 336, 1930.

## A COMPARISON OF TESTS FOR INSULIN SENSITIVITY\*

PAUL H. BURGERT, M.D., WALTER H. NADLER, M.D., AND RUTH STOTT, A.B.  
CHICAGO, ILL.

THE use of insulin early disclosed variations in response. Radoslav<sup>1</sup> in 1924 studied the effect of 20 units subcutaneously in 29 fasting patients with diabetes as well as in normal individuals and patients with renal glycosuria, and proposed this test for differential diagnosis. The fall in blood sugar ranged between 34 and 77 per cent, and was most marked when the original blood sugar was high. Intravenous insulin, 10 units, was given in several cases as a check. One patient with diabetes exhibited resistance to insulin; 20 units subcutaneously and 10 and 20 units intravenously produced little effect even though the original level was high. Falta and his associates continued these studies and in his monograph published in 1936, Falta<sup>2</sup> based his discussion on the classification of diabetic patients as sensitive and resistant to insulin. In 362 patients, 34.2 per cent were sensitive, 51.4 per cent were resistant, and 14.4 per cent were intermediary, but resembled the resistant persons. Obesity was more than twice as frequent in resistant and intermediate as in sensitive patients.

MacBryde<sup>3</sup> in 1936 reported similar tests to be of value as an index to dietary management. Fifteen patients fell into one of two groups: insulin sensitive patients failed to gain tolerance with a higher carbohydrate diet, while relatively resistant cases gained tolerance when the carbohydrate allowance was increased. Himsworth<sup>4</sup> in 1936, using an insulin-glucose test and examining both arterial and venous blood sugar samples, also obtained two distinct types of curves, but reported contrary conclusions. Classified according to his method, insulin sensitive patients were able to utilize large increases in carbohydrate without additional insulin, whereas insensitive persons were unable to do so. The contradiction in results reported by these writers has, as stated by Wilder<sup>5</sup> who has reviewed the subject in detail, only added to the existing confusion.

Our experience with the Himsworth test in 9 patients<sup>6</sup> had revealed striking differences between typical curves of two types and had suggested that intermediate curves might be obtained. In general, the clinical conclusions of Himsworth as to the effect of dietary changes were supported. In the present study 14 patients who had been classified as to insulin sensitivity by the Himsworth method were tested by the intravenous insulin method of MacBryde. Subsequently 13 of these patients were examined by the subcutaneous insulin method.

### PROCEDURE

The test described by Himsworth was used with only slight modification. Capillary and venous blood samples were drawn from fasting patients believed to be stabilized after months or years of treatment. Regular insulin, 5 units per square meter of body surface, as determined from a nomogram, was then in-

\*From the Department of Medicine, Northwestern University Medical School.  
Received for publication, December 31, 1938.

jected intravenously, immediately afterwards 30 gm of dextrose per square meter of body surface were given by mouth. Venous and capillary blood samples were obtained at intervals of five, ten, twenty, thirty, forty, fifty, and sixty minutes after ingestion of dextrose.

The insulin tests of Radoslav were performed as described by MacBryde. With the subcutaneous method, specimens of capillary blood were examined hourly for four hours after the injection into the fasting patient of 1 unit of insulin per 10 pounds of body weight. The per cent of fall in blood sugar from the fasting level to the lowest point observed was computed. With the intravenous method,  $\frac{1}{15}$  unit of insulin per kilogram body weight was given in the fasting state, and capillary blood samples were drawn every fifteen minutes for one and one half hours.

Blood sugar determinations were made by means of a modification of the Folin Wu micromethod. Duplicate tests were made in nearly all instances.

TABLE I

PATIENT	SEX	AGE	WEIGHT PER CENT NORMAL	DURATION	CARBOHY- DRATE ALLOW- ANCE (GRAMS)	INSULIN	ASSOCIATED FINDINGS
1 (MG)	M	18	-7	5 years	160	40P+10	
2 (BA)	M	16	Normal	64 years	150	40P+10	
3 (HK)	F	26	-10	74 years	150	40P+10	
4 (HW)	M	29	-20	9 months	215	15P	
5 (BB)	F	32	+10	4 years	15	20P	
6 (HH)	M	18	+20	5 years	150	40P+20	
7 (FS)	F	14	Normal	5 years	18	40P+20	
8 (TS)	F	33	(	8 years	110	10P	
9 (MR)	F	35	-25	7 years	165	10P	Constitutional inferiority
10 (G)	F	34	5	5 years	165	16P	Hay fever
11 (W)	F	9	5	14 years	150	35P	
12 (AG)	F	40	-5	14 years	160	50P+12	
13 (FM)	F	57	-15	5 years	100	40P	Hypertension, hay fever
14 (HS)	M	57	Normal	8 years	110	10P	Arteriosclerosis, angina

## RESULTS

Clinical data concerning the patients studied appear in Table I. Their ages ranged from 16 to 57 years. In all patients except 2 the diabetes had been treated for at least three years. Only 2 patients were overweight, 6 were underweight within 10 per cent, and 3 over 15 per cent. Definite clinical arteriosclerosis was present in only 2 cases. Two patients gave a history of recurrent hay fever. Patient 7 was a daughter of Patient 8. The carbohydrate allowances ranged from 110 to 215 gm and averaged 154 gm. The diets were adequate in protein content and in calories. All the patients had been treated with protamine zinc insulin for at least six months.

Classification according to the insulin glucose method of Himsworth and to the subcutaneous and intravenous insulin tests of MacBryde is listed in Table II.

The Himsworth test showed striking differences between insulin sensitivity and insensitivity in the majority of cases. In several instances the arterial and venous blood sugar curves were not typical in every respect, but they showed general characteristics either of sensitivity or insensitivity. In 14 patients the Himsworth method showed 5 sensitive and 9 insensitive.



TABLE II

PATIENT	HIMSWORTH			MAC BRYDE: SUBCUTANEOUS				MAC BRYDE: INTRAVENOUS			
		BLOOD SUGAR BEFORE TEST (MG. %)		FASTING BLOOD SUGAR (MG. %)	LOWEST BLOOD SUGAR	PER CENT FALL		FASTING BLOOD SUGAR (MG. %)	LOWEST BLOOD SUGAR	PER CENT FALL	
1	Sensitive	-	Sensitive	119.0	25.0	79.0	Sensitive	113.0	30.0	73.0	
2	Sensitive	150	Sensitive	338.5	49.5	85.0	<i>Borderline</i>	69.4	52.9	24.0	
3	Sensitive	206	Sensitive	264.0	77.0	71.0	Sensitive	94.4	30.7	66.0	
4	Sensitive	173	Sensitive	156.4	46.6	70.8	Sensitive	105.2	34.4	67.0	
5	Sensitive	143	<i>Insensitive</i>	212.2	113.4	47.0	Sensitive	196.6	92.6	53.0	
6	Sensitive	126	<i>Sensitive</i>	214.4	40.0	81.0	<i>Borderline</i>	250.0	186.9	25.0	
7	Insensitive	169	<i>Borderline</i>	249.0	114.2	54.0	Sensitive	84.5	42.8	49.0	
8	Insensitive	77	-	-	-	-	Sensitive	46.2	15.2	67.0	
9	Insensitive	60	Insensitive	75.5	59.9	20.0	Sensitive	74.0	43.5	41.0	
10	Insensitive	90	Insensitive	105.2	50.2	52.0	Sensitive	107.5	53.0	51.0	
11	Insensitive	126	<i>Sensitive</i>	229.5	79.4	65.0	Sensitive	113.0	49.5	56.0	
12	Insensitive	54	Sensitive	61.0	23.7	61.0	Sensitive	197.0	47.8	75.0	
13	Insensitive	140	Insensitive	171.5	97.4	43.0	<i>Borderline</i>	107.0	72.8	32.0	
14	Insensitive	116	Insensitive	143.0	119.0	16.8	Insensitive	148.5	130.5	8.2	

Classification according to the insulin tolerance tests (columns 2 and 3) is based on the percentage of fall in blood sugar found by MacBryde and reported in 15 cases. (In cases of insulin sensitivity he observed a fall of 61 to 75 per cent by the subcutaneous and of 34 to 45 per cent by the intravenous method. In relatively resistant cases a fall of 32 to 53 per cent was found with the subcutaneous and of 12 to 22 per cent with the intravenous test. Percentages lying between these ranges are called *border line*.)

With the insulin tolerance tests used by MacBryde, interpretation and classification were often difficult because of the lack of a definite point of demarcation between the percentages of fall in blood sugar of sensitive and relatively resistant cases. The ranges of percentage fall in blood sugar reported by MacBryde in 15 cases were arbitrarily chosen as a standard of differentiation. Percentages lying between these levels were classed as border line. In our series the intravenous method tended to demonstrate sensitivity to insulin in most cases. In the 14 patients tested there was only one patient (No 14) who was insulin resistant. This patient showed a much greater degree of resistance than any other reported by MacBryde and evidently was similar to the one patient found by Radoslav. *In the 14 patients classified according to the intravenous MacBryde method, 10 were insulin sensitive, 3 were border line, and one was insensitive. Two of the borderline patients veered toward sensitivity and one toward insulin resistance.* The definitely resistant case was also resistant with the other methods. One Himsworth sensitive person (No 2) showed, with the intravenous MacBryde test, a drop in blood sugar level only slightly above the range reported by MacBryde in 3 resistant patients. *Of 9 Himsworth insensitive patients, sensitivity to insulin was indicated in 6 by the intravenous MacBryde method.*

The subcutaneous insulin tolerance test was compared with the Himsworth test in 13 patients. *With the subcutaneous MacBryde method, 7 patients were sensitive, 5 were insensitive or "relatively resistant," and one was border line verging on resistance. Of the 5 Himsworth sensitive patients, 4 were sensitive according to the subcutaneous MacBryde method, while one was insensitive. Of 8 Himsworth insensitive persons, 4 were likewise insensitive by means of the subcutaneous MacBryde test, one borderline patient was relatively resistant, 3 patients were sensitive.*

A comparison of the results of intravenous and subcutaneous insulin tolerance tests was made in 13 patients. As previously indicated the former method showed 9 sensitive, 3 border line, and one very resistant while the latter showed 7 sensitive, 5 insensitive, and one border line. *Of the 9 patients sensitive to insulin by the intravenous method, only 5 showed comparable sensitivity to the subcutaneous test. Three patients insensitive according to the subcutaneous test were definitely sensitive with the intravenous method, one patient was insensitive with the subcutaneous and border line, veering on resistance, with the intravenous test.*

In general, although all three tests usually indicated sensitivity in patients who had experienced many insulin reactions, there were exceptions to this rule, notably Patient 12. This patient had a severe, unstable diabetes of 14 years' duration, insulin reactions were frequent. The Himsworth test disclosed insensitivity to insulin, while the MacBryde tests indicated sensitivity. One patient, No 14, exhibited undoubted resistance to insulin by all tests. Disagreement with the Himsworth test by the other tests was most frequent in cases of Himsworth insensitiveness. While disagreement between the two MacBryde tests seemed due mainly to uncertainty as to the degree of relative resistance, other differences are not so readily explained. For example, Patient 9, an undernourished woman

of 35 years with constitutional inferiority, was insulin insensitive both by the Himsworth and subcutaneous MacBryde methods but definitely sensitive with the intravenous MacBryde test.

### DISCUSSION

The following factors might influence the results obtained: the fact that the tests were made on ambulatory patients; the general condition at the time of the test; the time intervals between examinations; the carbohydrate content of the preceding diet; the use of protamine zinc insulin, including an injection twenty-four to twenty-six hours before each test; and the level of the fasting blood sugar.

Most of the patients had previous hospital education. Although in several patients the diabetes was unstable, in all of them the disease had for months been under good, or fairly good, control. They were apparently well and without complaints at the time of the tests. During the weeks or months between tests, the diet and insulin dosage were practically unchanged. The carbohydrate content of the diet, within the range of 110 and 215 gm., employed in this group of patients, was apparently not a factor. Patient 7, a juvenile with diabetes, receiving 183 gm. of carbohydrate, was Himsworth insensitive as was her father; the subcutaneous MacBryde test gave a borderline response, while the intravenous method indicated sensitivity to insulin.

The use of protamine zinc insulin up to twenty-four hours preceding the tests may undoubtedly have influenced the results, predisposing to sensitivity, in a number of cases. This factor was studied in three patients. In Patient 1, sensitive to all three tests, a fasting blood sugar of 67 mg. per 100 c.c. fell 53.8 per cent to 31 mg. after ninety minutes of continued fasting. Patient 4, however, also sensitive to all three tests, had a fasting blood sugar level of 99 mg. per 100 c.c., and one hour and thirty minutes later a fasting level of 120 mg. Patient 12, Himsworth insensitive and MacBryde sensitive, showed a drop of only 12 per cent (from 220 to 193 mg. per 100 c.c.) after one hour and thirty minutes of continued fasting. It seems evident that the use of protamine zinc insulin prior to examinations could not be entirely responsible for the conflicting results of the various tests used. In particular it is difficult to explain how a blood-sugar lowering effect, possibly prolonged for more than twenty-four hours, could be a factor in promoting the insulin-resistant responses.

The fasting blood sugar level is undoubtedly an important, if not the determining, factor in influencing the percentage of fall following the injections of insulin. This was clearly recognized by Radoslav. The high percentage of drop in blood sugar noted in Patients 2, 3, and 6, after the subcutaneous injection of insulin, is probably explained on this basis. In Patient 6, however, the intravenous MacBryde test revealed no comparable fall even though the fasting blood sugar was high.

Holecomb and Holecomb<sup>8</sup> have used the MacBryde test as a basis of classification in 7 patients. Six patients with high fasting blood sugars showed a precipitous drop; on computing the percentages of fall the variations ranged

from 45 to 75 per cent, with 3 under 55 per cent. The one patient considered insulin "tolerant," or relatively resistant, showed a drop of 60 per cent (from about 160 to 60 mg per 100 cc). It is impossible to accept this case as one of "resistance" to insulin.

It seems plain from our data, and it is recognized by Faltz, that borderline cases occur when insulin tolerance tests are used. Klatskin<sup>9</sup> applied the subcutaneous MacBride test to 50 diabetic patients and found wide variations in the percentage fall of blood sugar, ranging from 30 to 85 per cent. Using a drop of 60 per cent as a dividing line, no natural cleavage between insulin sensitivity and resistance could be made out, and no significant relationship between sensitivity and response to high carbohydrate diets could be demonstrated. Soskin and Levine<sup>10</sup> have reported the reproduction in the same completely depancreatized dog, at different times, of both types of diabetes. Hepatic injury reversed the sensitive type of curve. They conclude that the significant difference between insulin sensitivity and relative resistance depends upon the functional capacity of the liver.

The Himsworth test has demonstrated insensitive types of curves in unusual cases of diabetes. One of our patients with acromegaly and diabetes (not included in this series) showed, before treatment of the diabetes, an intermediate curve with the general characteristics of insensitivity. Three weeks later, after treatment with diet and insulin and after irradiation, more typical insensitive curves were obtained. Flaum<sup>11</sup> reported that insensitive types of curves were obtained in the study of diabetes associated with acromegaly and suggested that the phenomenon of Himsworth insulin insensitivity when seen in diabetes uncomplicated by obvious endocrine dysfunction may possibly be due to hyperactivity of one or more of the endocrine glands. Marble<sup>12</sup> reported Himsworth insensitiveness in a diabetic requiring very large amounts of insulin without demonstrable explanation of this peculiarity. His patient had insulin reactions. Patient 12 in our series, although insulin insensitive, is an unstable diabetic person who has frequent reactions. According to Himsworth, the response to the test is not dependent upon liver function. The question arises whether this test is also reversible. Patient 12 was unmistakably insensitive on December 2, 1937. About ten months later the response was intermediate in that the arterial-venous differences had widened considerably, the curves were, however, still of the insensitive type.

de Wesselow and Griffiths,<sup>13</sup> instead of the Himsworth test, gave to a series of normal and diabetic patients 5 units of insulin intravenously and 50 gm of glucose by mouth, and tested capillary blood sugar in the fasting state and after thirty and sixty minutes. Results were expressed as the algebraic sum of the differences between the initial blood sugar and the values thirty and sixty minutes after the insulin injection. No evidence of the existence of two distinct types of diabetes was obtained. Sensitivity to the test depended to a large extent upon an adequate carbohydrate allowance in the diet. It was found by this test that resistant diabetic patients may become sensitive when put on a high carbohydrate ration, adequately controlled by insulin.

## CONCLUSIONS

1. Comparisons of classification as to insulin sensitivity or insensitivity between the Himsworth and the subcutaneous and intravenous MacBryde tests revealed no dependable correlation. In some patients all of the tests demonstrated definite evidence of marked insulin effect. Only one patient was unmistakably insensitive by all three methods. The subcutaneous and intravenous MacBryde tests did not agree in all instances.

2. The Himsworth and the insulin tolerance tests evidently do not give the same information. The former presents more striking and definite differences between insulin sensitivity and insensitivity. The latter methods present difficulties in appraising degrees of relative resistance; borderline cases occur. With the subcutaneous MacBryde test the greatest percentage fall in blood sugar usually occurs when the fasting level is high.

3. Classification as to insulin sensitivity or insensitivity should be made only in terms of the particular test used. The possible reversibility of the various tests is discussed. From the evidence available there appears to be no justification for the classification of diabetes as of two distinct types based solely on sensitivity or resistance to insulin.

## REFERENCES

1. Radoslav, C. S.: Ueber die Wirkung des Insulins auf den Blutzucker beim Menschen, *Wien. Arch. f. inn. Med.* 8: 395, 1924.
2. Falta, W.: *Die Zuckerkrankheit*, Berlin, Vienna, 1936, Urban & Schwarzenberg.
3. MacBryde, C. M.: Response to Insulin as an Index to the Dietary Management of Diabetes, *J. Clin. Investigation* 15: 577, 1936.
4. Himsworth, H. P.: Diabetes Mellitus: Its Differentiation Into Insulin-Sensitive and Insulin-Insensitive Types, *Lancet* 1: 127, 1936.
5. Wilder, R. M., and Wilbur, D. L.: Diseases of Metabolism and Nutrition: Review of Certain Recent Contributions, *Arch. Int. Med.* 59: 329, 1937.
6. Nadler, W. H.: Sensitiveness to Insulin and Optimal Diets in Diabetes, *Arch. Path.* 26: 395, 1938.
7. Nadler, W. H., Starr, P., and Tukey, G.: Glycemia as a Guide in the Treatment of Diabetes Mellitus: The Practicability of Routine Examinations of Small Effectively Preserved Specimens of Blood Drawn by the Patient, *Arch. Int. Med.* 36: 579, 1926.
8. Holcomb, B., and Holcomb, R.: Response of Insulin-Sensitive and Insulin-Tolerant Patients to Protamine Insulin, *Northwest Med.* 37: 36, 1938.
9. Klatskin, G.: The Response of Diabetics to a Standard Test Dose of Insulin, *J. Clin. Investigation* 17: 745, 1938.
10. Soskin, S., and Levine, R.: Experimental Production of Insulin-Sensitive and Insulin-Insensitive Types of Diabetes, *Abst., J. A. M. A.* 110: 768, 1938.
11. Flaum, G.: Insulin Sensitivity in Endocrine Disorders, *Abst., Am. J. Physiol.* 123: 67, 1938.
12. Marble, A.: Insulin Resistance: Report of a Case of Marked Insensitiveness of Long Duration Without Demonstrable Cause, *Arch. Int. Med.* 62: 432, 1938.
13. de Wesselow, O. L. V., and Griffiths, W. J.: Insulin Resistance in Diabetes Mellitus and the Effect of Dietary Carbohydrate, *Quart. J. Med.* 7: 17, 1938.

## AN UNUSUAL CASE OF AUTOAGGLUTINATION\*

K M WHIFFLER, PH D, H J GALLAGHER MD, AND C A STUART, PH D  
PROVIDENCE, R I

COLD agglutinins or autoagglutinins occur normally in many human sera<sup>1</sup> In various pathologic conditions, however, the titer may be greatly increased<sup>2</sup> and may interfere with the usual O A B and AB blood grouping tests<sup>3</sup> We encountered such a case in a routine typing for transfusion and have studied it in some detail

The patient, G C, a white female 38 years old was admitted to the hospital December 1, 1937, complaining of a severe cough, weakness and fever She had been ill for five days with symptoms of upper respiratory infection, headache, and malaise which became progressively worse On admittance the patient had a temperature of 102.4° F, a respiration and pulse rate of 25 and 100, respectively, and showed typical consolidation signs in the right lower lobe which were confirmed by x-ray A pericardial friction rub and general toxemia with cyanosis were also noted The white blood count was 14,150 with marked polymorphonuclear shift to left The red blood count hemoglobin blood chemistry, and urine were normal For thirteen days the temperature showed high septic spikes with corresponding increases in pulse and respiration A pneumococcus which could not be typed was twice found in the sputum but blood cultures were consistently negative Oxygen high fluid and symptomatic therapy were given The general toxemia increased with delirium and marked prostration On December 10 an x-ray examination showed an increased mottled density in both lower lung fields with no fluid, and an electrocardiogram revealed suggestive pericarditis and myocarditis and myocardial involvement A transfusion of 300 cc was given December 14 which was followed by a four day drop of the vital signs (temperature, respiration and pulse) A new rise in the vital signs occurred on Dec 21 with constant pneumonitis in the right and left lower lung fields with a diagnosis of unresolved pneumonia and possible encapsulated empyema which yielded no fluid when the area was tapped A second transfusion of 300 cc was given on December 27 without significant change in the course of the infection By January 15 the vital signs had dropped to normal with a marked gain in the patient's general condition and clearing of the lungs The remainder of the patient's course in the hospital was uneventful, except for occasional temperature spikes and associated arthralgia The patient was discharged March 13 much improved in strength and general condition

The first attempt at typing was made at room temperature using the patient's unwashed erythrocytes Agglutination occurred in both the anti A and anti B sera as well as in the saline control When the cells were washed twice

\*From the Biological Laboratory of Brown University and the Rhode Island Hospital Providence

Received for publication January 3 1939

with saline, however, they agglutinated only in anti-A serum and were subsequently subgrouped as A<sub>1</sub> type MN. The patient's serum agglutinated O, A<sub>1</sub>, and autologous cells at room temperature to a titer of 320. The reactions were completely reversible by warming to 37° C. A group A (A<sub>1</sub>) donor was selected whose cells were not agglutinated at 37°, but who reacted strongly with the patient's serum at 22°, and a successful transfusion of 300 c.c. was performed. Two weeks later a second transfusion from the same donor was given.

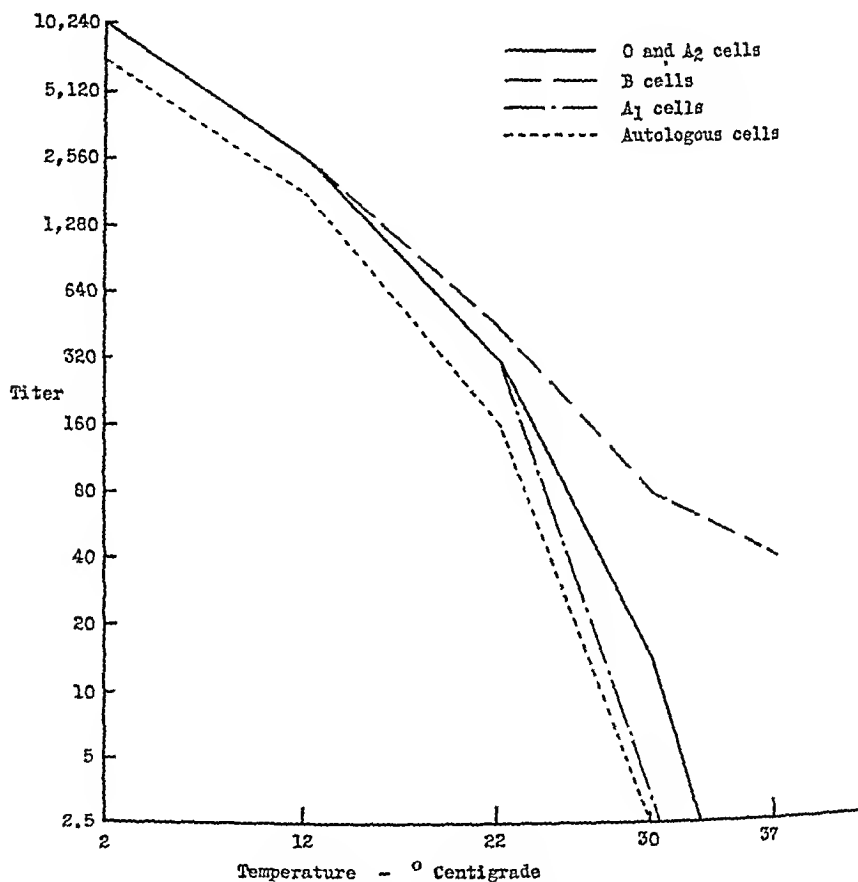


Fig. 1.—Average thermal curves for agglutination of O, A, B, and autologous cells by cold agglutinins of a human (G. C.) serum.

Blood samples were taken over a three months' period and the cold agglutinin titered at 2° with autologous, O, A, and B cells. All titers are expressed as the reciprocal of the highest final dilution of serum which gave agglutination. From 10,240 at the first bleeding on December 13, the titer dropped to 2560 after one week, 160 after three weeks, 20 after seven weeks, and 5 after three months. These and the following tests were done macroscopically using small serologic tubes and a final concentration of 0.15 per cent cells. Incubation times were those generally used at the various temperatures.<sup>1</sup>

Sera from the first and second bleedings were tested with autologous cells and with several O, A<sub>1</sub>, A<sub>2</sub>, and B cells at 37°, 30°, 22°, 12°, and 2°. Thermal curves for these cells are shown in Fig. 1. Titers for the autologous cells were

slightly lower than averages of the other  $A_1$  cells. Titers for O and  $A_2$  cells were the same, and at 30° the serum showed the properties of an  $\alpha$  agglutinin since the titer for O and  $A_2$  cells was fourfold that of the  $A_1$  titer. At 37° only the anti B isoagglutinin was effective.

TABLE I  
ADSORPTION AND ELUTION OF COLD AGGLUTININS

SERUM	AVERAGE 2° C COLD AGGLUTININ TITERS FOR					
	AUTOLOGOUS CELLS ( $A_1$ )	O CELLS	$A_1$ CELLS	B CELLS	RABBIT CELLS	SHEEP CELLS
Unadsorbed	1840	5120	5120	5120	5120	160
Adsorbed with						
Autologous cells	<10	320	40	240	2560	40
O cells	160	40	80	80	2560	80
B cells	160	160	80	40	2560	20
Rabbit cells	20	1280	140	1280	20	20
Sheep cells	1280	1280	1280	1280	2560	<10
Eluate from						
Autologous cells	1280	3840	2560	2560	2560	80
O cells	1280	3840	1280	2560	2560	80
B cells	1280	2560	2560	2560	2560	40
Rabbit cells	1280	1280	640	140	5120	20
Sheep cells	1280	1280	140	1280	2560	80

Although the autoagglutinin acts on cells of all groups and is usually considered nonspecific, specific qualities in this case were shown by adsorption. The patient's serum in a 1:5 dilution was adsorbed at 2° for three successive times with autologous A, O, B, rabbit and sheep erythrocytes and then tested at 2° with the various cells as shown in Table I. In some instances agglutinins were not completely removed, but in all cases titers after adsorption were lowest for the homologous cells used in the adsorption. Autoagglutinins for the patient's cells ( $A_1$ ) were completely removed after three adsorptions, yet the serum contained cold agglutinins for human O and B, sheep and rabbit cells and also, to a slight extent, for other  $A_1$  cells. Similar reactions occurred following adsorptions with O or B cells, except that the titer differences in the adsorbed sera were less marked. Landsteiner<sup>4</sup> at an early date showed that cold agglutinins which had been adsorbed at low temperatures could be released from the cell by warming to body temperature or higher. By this method, purified solutions of the cold agglutinins were prepared from the various cells. Cells from the first 2° adsorption of the patient's serum were washed once at 2° with saline to remove traces of serum and then eluted at 50° in saline and centrifuged at that temperature. Eluates prepared in this way from autologous A, O, B, rabbit, and sheep cells were tested at 2° with the various cells with the results given in Table I. All elution fluids agglutinated each kind of cell with which they were tested, and titers were much the same for a particular type of cell regardless of the source of the eluate. The eluates exhibited no group specificity and even agglutinated erythrocytes from rabbits and sheep, species not closely related to human beings, although these species do have common heterogenetic antigens.

#### COMMENT

This patient seems of interest because of the unusually high titer of the cold agglutinin (10,240). When the blood was allowed to clot at room temperature



and the serum taken off at room temperature, the titer was somewhat lower (2560), probably due to adsorption of some of the agglutinin. Maximum titer could be obtained only when the serum was removed at 37° or higher. The cells of the patient reacted as normal A<sub>1</sub> cells, except that they were slightly less sensitive to agglutination than most other A<sub>1</sub> erythrocytes. The adsorption at 2° also showed a difference between the patient's cells and other A<sub>1</sub> cells since after adsorption with autologous cells the patient's serum still reacted with other A<sub>1</sub> cells.

Other members of the patient's family were examined since there is some evidence for familial occurrence of the cold agglutinin.<sup>2</sup> The father, mother, brother, sister, and daughter were examined and none of the sera in 1:5 dilution possessed autoagglutinins. This would probably be expected since the potent agglutinins of the patient were only temporary and diminished rapidly after the time of the first test.

Although autoagglutinins do not seem to be functional at body temperature, they are important as interfering agglutinins in blood grouping tests as usually done. The importance of the cross match of recipient's serum with donor's cells, and donor's serum with recipient's cells should be emphasized. When unusual reactions occur, such as agglutination of O cells at room temperature, the reaction is probably due to a cold- or autoagglutinin, and a correct typing and match will be given when the test is made at 37°. One other possibility should be kept in mind, however, and that is the case Morzycki<sup>5</sup> has described in which the blood (A<sub>1</sub>) was deficient in the human species antigen and the serum contained agglutinins for O and A<sub>2</sub> cells which were functional at 37°. Presumably a group O donor would not be compatible for a blood of this sort.

#### REFERENCES

1. Kettel, K.: Studien über die Frage die Kalteagglutination des Blutes bei Menschen, *Acta path. et microbiol. Scandinav.* 5: 306, 1928.
2. Boxwell, W., and Bigger, J. W.: Autohemagglutination, *J. Path. & Bact.* 34: 407, 1931.
3. Manheims, P. J., and Brunner, E. K.: Faulty Blood Grouping Due to Auto-agglutinins: An Unusual Case, *J. A. M. A.* 101: 207, 1933.
4. Landsteiner, K.: Ueber Beziehungen zwischen dem Blutserum und den Körperzellen, *München. med. Wchnschr.* 50: 1812, 1903.
5. Morzycki, J.: Ueber das Wesen der Agglutinine gegen das Menschenblut der O-Gruppe, *Ztschr. f. Immunitätsforsch. u. exper. Therap.* 84: 80, 1934.

## GLUCONIC ACID AS A URINARY ACIDIFYING AGENT IN MAN\*

HARRY GOLD, M.D. AND HILFE CIVIN, M.S.  
NEW YORK, N. Y.

WITH THE ASSISTANCE OF CHARLES SALZMAN, B.S.

THE fate of gluconic acid in the animal organism is a matter which is not fully established. In 1899 Salkowski<sup>1</sup> administered about 7 gm. of sodium gluconate daily to a rabbit for three consecutive days. Pentoses did not appear in the urine, and inasmuch as the urine during this time was strongly alkaline and contained little oxalic acid, he concluded that the gluconic acid was completely oxidized. In 1902 Mayer<sup>2</sup> published experiments in which he, too, was unable to recover any gluconic acid from the urine of rabbits after the oral administration of a very large dose, 71 gm. of sodium gluconate per kilogram of body weight. In 1905 Banngarten<sup>3</sup> reported that 10 gm. doses of sodium gluconate in small normal, and depancreatized dogs and 10 to 20 gm. doses in diabetic persons were also completely oxidized. In these experiments the urine failed to develop optical rotation or reducing power and failed to yield combinations with phenylhydrazine. Several years later (1930), however, Hermann<sup>4</sup> reported a contrary result. He described an experiment in which he recovered 75 per cent of an oral dose of about 5.4 gm. of gluconic acid per kilogram of body weight in the urine of a rabbit. He suggested that this small recovery might be due to destruction of gluconic acid by microorganisms in the bowel prior to absorption. This, however, could not explain the results of experiments by other authors in which only small amounts of gluconic acid were recovered in the urine after the drug was administered subcutaneously.

After subcutaneous injections of sodium gluconate in doses of the order of 7 gm. per kg. in the rabbit, Mayer<sup>2</sup> recovered about 10 per cent in the urine, he stated that it existed in the urine in the form of saccharic acid. In 1911 Schott<sup>5</sup> confirmed the results of Mayer in part in that he also found that after subcutaneous large doses of sodium gluconate, total amounts of about 15 gm., a substance appeared in the urine which he believed, however, was not saccharic acid, but gluconic acid, and the amounts recovered represented from about 15 to 50 per cent of the dose injected.

Direct evidence concerning the fate of that part of gluconic acid which is not recovered in the urine does not exist. Schwarz<sup>6</sup> in 1903 showed that the oral administration of sodium gluconate sometimes reduced the excretion of acetone bodies in the urine and breath more effectively than even larger doses of glucose.

\*From the Department of Pharmacology, Cornell University Medical College, New York.  
Received for publication January 11, 1939.

in diabetic patients. Baer and Blum<sup>7</sup> in 1911 failed to find diminished excretion of acetone bodies in phlorhizinized dogs and referred to this as an indication that gluconic acid is not converted to saccharic acid which they found does reduce the excretion of acetone bodies. Paderi<sup>8</sup> in 1918 concluded that gluconic acid is transformed into glucose because in his experiments the incubation of the acid with liver increased the glucose content, and the administration of gluconic acid to fasting rabbits increased the glycogen content of the liver.

The preponderance of evidence, therefore, seemed to indicate that while varying amounts of gluconic acid may appear in the urine after subcutaneous injection, little or none of even a large dose of it appears in the urine after its oral administration. It should be noted that the experiment in which some gluconic acid was found in the urine after the drug was administered orally involved a dose equivalent to about 300 gm. for an average adult of about 60 kg. Since only so little spilled over in the urine after such a massive amount, it cannot be stated whether any of it would be found in the urine after total doses of about 25 gm. a day which are given to human beings. In contrast to the above evidence on the conversion of gluconic acid into sugar and the failure of most investigators to isolate it in the urine after exceedingly large oral doses. Hermann<sup>9</sup> expressed the opinion that gluconic acid is an exception among the common organic acids in that it is not oxidized in the organism, although his experiments<sup>4</sup> presented no direct proof of this view.

Hermann<sup>9</sup> stated that acid soluble phosphate calculi placed in the bladder of the rabbit, were gradually reduced in size until they passed through the urethra, as the result of the daily feeding of gluconic acid for several weeks. How such calculi fared without gluconic acid was not stated. In these experiments massive doses of gluconic acid were given, 20 c.c. of a 5 per cent solution two to three times daily to a rabbit, equivalent to from 60 to 90 gm. or 1,200 to 1,800 c.c. of a 5 per cent solution of gluconic acid daily for the average man. He also stated that alkaline urine becomes acid after oral doses of gluconic acid but presented no controlled observations concerning this phenomenon. It should be noted that this observation is in conflict with that of Salkowski<sup>1</sup> who found that a dose of 7 gm. of sodium gluconate daily for three days left the urine of the rabbit strongly alkaline.

The papers of Hermann appear to have served as a basis for a recent report on gluconic acid and the delta lactone of gluconic acid as a urinary acidifying agent by Sisk and Toenhart.<sup>10</sup> They reported the effect on the pH of the urine in 7 human subjects, some with an infected and others with an uninfected urinary tract. They concluded that gluconic acid is a satisfactory urinary acidifying agent in man, and that its effects disappear rapidly so that frequent doses are necessary to maintain the low pH, namely, 4 to 6 gm. at intervals of two to four hours. They administered single doses of from 3 to 9 gm. every two hours, amounting to from 12 to 45 gm. daily, and stated that the minimum pH was usually reached in from one to three hours. This signifies, from the manner in which the doses were given, a minimum pH after total doses of from 3 to 18 gm. of gluconic acid.

In 4 of the 7 cases studied by Sisk and Toenhart there were no controls the first pH being determined one hour after the first dose of the drug. In 2 cases the control consisted of one pH value before each day's treatment. When these results are examined, using in each case the first pH of the day as a control,\* one finds that of 35 such days the urine was most alkaline in the first specimen on twenty days, and most alkaline in at least one specimen during the course of the treatment on fifteen days. It is well known that the pH shows wide fluctuations throughout the day, and the control course under the conditions of these experiments was reported in only one patient and in that case (Case 2), the pH averaged 6.4 (range 5.7 to 6.7) in the first two day control period, and 6.1 (range 4.9 to 6.9) in the first two day treatment period. Such a difference is also seen when the two control days are compared with each other, namely 6.2 for the first day and 6.5 for the second day, and in this patient in a series of eight treatment days with the same daily doses the average of the daily pH values varied irregularly from 5.1 to 6.1. The pH of the urine taken at 9 A.M. (one hour after the first dose) on eight different days varied in this individual from 5.2 to 6.9. In then Case 5, for example the first specimens of urine of the two day period of gluconic acid treatment averaged 6.7 while the first specimens of the four day period of ammonium nitrate treatment averaged 5.6. These illustrations suffice to indicate the danger of isolated pH values as controls. These results, therefore, do not supply sufficient proof that gluconic acid is a useful urinary acidifying agent. The present experiments were undertaken to re-examine this question.

TABLE I

COMPARISON OF pH VALUES OF URINE WITH GLASS AND QUINHYDRONE ELECTRODES

SUBJECT	GLASS	QUINHYDRONE*	DIFFERENCE
T	4.88	5.02	0.14
H	5.15	5.34	0.19
M	5.00	5.11	0.16
J	5.44	5.54	0.10
T	4.82	4.92	0.11
M	5.04	5.17	0.13
J	5.42	5.50	0.08
H	5.02	5.06	0.04
M	4.86	5.02	0.16
J	5.27	5.29	0.02
C	5.21	5.11	0.10

\*The readings were made promptly after the quinhydrone was added.

## EXPERIMENTAL

More than 350 specimens of urine were examined for specific gravity and pH. The pH was determined by the glass electrode in one series, and by the quinhydrone electrode in the other series. Eleven specimens of urine examined by both methods showed uniformly slightly higher values with the quinhydrone electrode (Table I). The latter is not quite as satisfactory as the glass electrode for urine because of the tendency to drifting if there is delay in making the reading.

\*The first pH in the records was either before treatment or after one dose of gluconic acid.

In one group of experiments 5 normal subjects were used, and a determination was made of the effect of a large dose of gluconic acid on the pH of the urine secreted during the first four-hour period after the dose. The plan included the following: constant breakfast at about 8 A.M. (such as coffee, or roll and coffee); the bladder emptied at 9 A.M. and voiding discarded; a glass of water at 9 A.M. (in controls), or 15 gm. of the gluconic acid\* washed down with the glass of water (on the treatment days); no food, drink, or voiding until 1 P.M. In all, about 70 daily specimens of urine were examined during this part of the work. The urine was examined as soon after voiding as it reached room temperature (fifteen or twenty minutes).

In the second group of experiments an attempt was made in another way to determine the effect of gluconic acid on the acidity of the urine, namely, to compare the average as well as the range of several pH values on one or more control days with those on one or more treatment days. Observations were made on 16 persons,† 8 of whom were confined to bed in the hospital, and 8 of whom were ambulant. In 7 of the bed patients there were various urologic conditions with urinary infection. These patients were selected in part because of the high pH values of their urine before treatment, which served as a more satisfactory control for the effects of an acidifying agent. The diets of the ambulant patients, as well as their urinary volumes, varied considerably, but in the case of the bed patients they were the regular ward diets of the hospital, which are more constant. The daily urinary output in these patients varied between approximately 1,500 and 3,000 c.c., except in one patient (Case 2) in whom the daily urine volume was only about 900 c.c. The delta lactone was administered in 5 gm. doses at two-hour intervals up to total doses of from 15 to 25 gm. daily, and subsequently in 10 gm. doses every two hours up to total doses of from 20 to 50 gm. daily. In one case a single dose of 15 gm. was given. In another case 2 doses of 25 gm. each were given at two-hour intervals. In a few instances the delta lactone was dissolved in water and diluted to an approximately 2 to 3 per cent solution for administration. This proved rather irritating to the mouth, however, and in the majority of instances it was found more satisfactory to take it as the dry powder followed by a glass of water. The pH was determined with the quinhydrone electrode in this series. The specimens of urine were collected at two-hour intervals from 7 A.M. to 5 P.M., both on control days and treatment days, the specimens being kept on ice, and the pH values determined in less than an hour after collection. The specific gravity of every specimen was also determined. Since most of the subjects received abundant fluids, the specific gravity of the urines was fairly low (1.002 to 1.021) and appeared to vary without relation to the pH.

\*This was given as the delta lactone. It was supplied as the preparation of gluconic delta-lactone by Chas. Pfizer & Co., Inc.

†We are deeply indebted to Dr. Charles G. Child of the Department of Surgery for making available to us 7 urologic patients as subjects, and for his help in establishing the necessary routine in these cases; also to Dr. Ade T. Milhorat for his patient who was controlled on a strictly constant diet in connection with his work on progressive muscular dystrophy.

The following is a protocol of a typical experiment (Patient 2)

DATE	TIME OF SPECIMEN	7	9	11	1	5
<i>Control Days</i>						
10/12/38	pH		7.0	7.0	6.7	7.0
	Specific gravity	-	1.005	1.006	1.004	1.004
10/13/38	pH	6.9	7.6	6.9	7.5	7.1
	Specific gravity	1.008	1.012	1.012	1.012	1.012
<i>Treatment Days</i>						
10/14/38	pH	9.2	8.8	8.8	8.9	9.7
	Specific gravity	1.027	1.009	1.010	1.010	1.010
Dose of drug at		7	9	11	1	
10/15/38	pH	8.7	8.6	9.1	8.9	6.6
	Specific gravity	1.020	1.021	1.009	1.006	1.004
Dose of drug at		7	9	11	1	3
<i>Control Day</i>						
10/19/38	pH	9.4	8.9	8.4	8.7	8.8
	Specific gravity	1.006	1.008	1.004	1.002	1.006
10/20/38	pH	7.6	7.6	7	7.2	6.9
	Specific gravity	1.010	1.007	1.008	1.010	1.008
<i>Treatment Day</i>						
10/21/38	pH	7.0	7.1	7.0	7.5	6.9
	Specific gravity	1.008	1.008	1.010	1.010	1.006
*Dose of drug at		7	9	11	1	

\*Dose of drug was 5 gm during first two days of treatment and 10 gm during the third day. It was discontinued because of nausea and diarrhea.

#### RESULTS

The results obtained in the first series of experiments are shown in Table II. The indications are unequivocal in showing that the urine secreted during the first four hour period after a very large dose of the delta lactone of gluconic acid is more acid than control specimens obtained under similar conditions. The pH of the gluconic acid periods never reached the high values of the controls, although they did not go far below the lowest values of the controls. That the urine resists acidification below the lowest normal pH values has been observed by others in connection with other urinary acidifying agents (Johnston, 1932<sup>11</sup>). Three of the 5 subjects developed abdominal cramps and diarrhea. In one patient vomiting occurred. The specific gravity of the urine showed no consistent relationship to the changes in the pH.

The essentials of the results in the second series of experiments are summarized in Table III. Since the values after the 10 gm doses were similar to those after the smaller ones, they have been considered together in the table. In the control periods the subjects had been without drug for at least twenty-four hours, and in the values of the treatment period the last specimens included are those taken no later than two hours after the last dose of the drug.

In 8 of the 16 patients, the urine became more acid, and in the other half it became more alkaline during the period of treatment with gluconic acid. The failure of this series to reveal clearly the acidifying action of gluconic acid may

TABLE II

EFFECT OF DELTA LACTONE OF GLUCONIC ACID ON PH<sup>§</sup> OF URINE SECRETED DURING FIRST FOUR HOURS AFTER A DOSE OF 15 GM.

SUBJECT T	pH		SPECIFIC GRAVITY		SUBJECT M	pH		SPECIFIC GRAVITY	
	CONTROLS	AFTER DRUG	CON- TROLS	AFTER DRUG		CONTROLS	AFTER DRUG	CON- TROLS	AFTER DRUG
12/3	6.0	-	1.007	-	12/2	7.0	-	1.010	-
12/20	5.9	-	1.008	-	12/3	6.2	-	1.017	-
12/19	5.8	-	1.020	-	12/19	5.7	-	1.017	-
12/21	5.6	-	1.012	-	12/21	5.6	-	1.023	-
12/2	5.6	-	1.014	-	12/29	5.4	-	1.024	-
12/1	5.2	-	1.011	-	12/30	5.4	-	1.026	-
12/6	4.9	-	1.010	-	12/20	5.4	-	1.016	-
1/5	-	4.9	-	1.016	12/1	5.2	-	1.026	-
1/9	-	4.9	-	1.007	12/22	-	5.2	-	1.020
12/9	-	4.9	-	1.014	12/9	5.1	-	1.016	-
12/7	-	4.8	-	1.016	12/28	-	5.1†	-	1.020
12/10	-	4.8	-	1.010	12/27	-	5.0*	-	1.023
					12/6	-	5.0	-	1.025
					12/7	-	5.0	-	1.025
					12/8	-	4.9	-	1.018

SUBJECT J	pH		SPECIFIC GRAVITY		SUBJECT H	pH		SPECIFIC GRAVITY	
	CONTROLS	AFTER DRUG	CON- TROLS	AFTER DRUG		CONTROLS	AFTER DRUG	CON- TROLS	AFTER DRUG
12/1	7.2	-	1.018	-	12/2	5.2	-	1.024	-
12/30	6.8	-	1.024	-	12/16	5.2	-	1.024	-
12/3	6.2	-	1.024	-	12/19	5.2	-	1.022	-
12/16	5.9	-	1.019	-	12/20	5.2	-	1.023	-
12/27	5.8	-	1.016	-	12/6	-	5.2*	-	1.026
12/2	5.8	-	1.015	-	12/1	5.1	-	1.027	-
12/9	5.5	-	1.018	-	12/28	5.1	-	1.018	-
12/19	5.5	-	1.020	-	12/29	-	5.1†	-	1.016
12/22	-	5.5*	-	1.022	12/9	-	5.1*	-	1.026
12/20	5.5	-	1.020	-	12/22	-	5.1*	-	1.028
12/21	5.5	-	1.018	-	12/3	5.1	-	1.022	-
12/28	-	5.5‡	-	1.020	12/17	5.1	-	1.017	-
12/29	5.4	-	1.020	-	12/21	5.1	-	1.019	-
12/6	-	5.4	-	1.016	12/8	-	5.0	-	1.024
12/23	-	5.4	-	1.018	12/23	-	5.0*	-	1.031
12/7	-	5.4	-	1.018					
12/8	-	5.3	-	1.020					

SUBJECT C	pH		SPECIFIC GRAVITY	
	CONTROLS	AFTER DRUG	CON- TROLS	AFTER DRUG
12/23	7.1	-	1.019	-
12/22	6.9	-	1.011	-
12/29	6.3	-	1.025	-
12/9	5.9	-	1.010	-
12/30	5.9	-	1.020	-
1/5	-	5.4	-	1.019
1/6	-	5.3	-	1.012
12/8	5.2	-	1.012	-
12/16	-	5.2	-	1.005
12/21	5.1	-	1.012	-

\*Diarrhea caused by the drug.

†Dose only 10 gm.

‡Vomited within 1 hr. 15 min. after dose.

§The pH in this series was determined by the glass electrode.

in part be due to the smaller single doses and to the masking effect of diet during the day.

Eleven of the 16 patients developed diarrhea with or without nausea at some time in the course of the use of the gluconic acid; in 6 of these it occurred with the 5 gm. doses.

TABLE III

No	Subject	Sex	Age	Diagnosis	Infected Urine	Total Daily Dose of Lactone (gm.)			No of Control Days	No of Sickens in Controls	No of Sickens in Patients	pH Determinations				Average in pH during Treatment	Remarks	
						First Day (3 gm doses each)	Second Day (5 gm doses each)	Third Day (10 gm doses each)				Range in Controls	Range After Dose	Average in Controls	Average After Dose			
1	M	M	4	Renal calculus	+	25	25	50	4	24	15	15	8.1-6.4	9.7-6.2	6.9	7.1	+0.2	Diarrhea after 50 gm
2	M	M	30	Pyonephrosis and nephritis	+	25	25	50	4	10	14	14	9.0-6.9	9.7-6.6	7.6	8.1	+0.5	Diarrhea and nausea after 25 gm
3	F	F	26	Hypertension	+	25	25	50	4	14	13	13	7.7-6.7	7.0-5.7	7.5	6.6	0.9	Diarrhea after 0 gm
4	F	F	43	Renal calculus	+	25	25	50	4	18	15	15	7.5-6.5	7.4-6.7	7.2	7.1	0.1	Diarrhea after 0 gm
5	M	M	70	Polymyositis of blood der hydromyositis	+	-	-	50	2	9	-	-	7.6-6.0	7.1-6.5	6.9	6.8	0.1	Diarrhea after 20 gm
6	M	M	30	Intercurrent of blood der prolate and ludney	+	25	25	-	2	10	10	10	8.6-6.4	8.2-6.9	7.7	7.8	+0.1	Diarrhea after 20 gm
7	M	M	71	Bleeding hypertrophy of prostate transurethral resection	+	-	-	50	2	10	-	-	8.6-6.4	-	7.0	7.1	+0.1	Diarrhea and nausea after 40 gm
8	G	M	24	Normal	0	20	-	-	1	4	4	4	7.1-6.1	7.2-6.0	6.7	6.9	+0.2	Diarrhea after 20 gm
9	F	F	27	Normal	0	25	-	-	2	5	-	-	7.2-6.2	6.8-6.1	6.8	6.5	+0.7	Diarrhea after 15 gm
10	F	M	41	Normal	0	15	20	20	1	4	11	11	5.8-5.3	6.2-5.4	5.6	5.7	+0.1	Diarrhea after 15 gm
11	M	M	10	Progressive muscular dystrophy	0	15	25	50*	2	7	9	9	7.2-6.1	6.7-5.5	6.6	6.0	0.6	Diarrhea after 25 gm
12	F	F	7	Normal	0	20	20	-	3	10	10	10	7.3-5.2	6.2-5.0	6.2	5.2	1.0	-
13	M	M	30	Normal	0	-	-	15 (1 dose)	4	4	2	2	5.6-5.2	5.8-5.3	5.4	5.6	+0.2	-
14	G	M	41	Normal	0	15	-	-	1	2	3	3	-	5.4-5.0	5.5	5.2	0.1	-
15	M	M	52	Normal	0	15	-	-	1	4	-	-	6.5-5.3	5.4-5.0	5.8	5.2	0.6	Diarrhea after 15 gm
16	F	F	59	Normal	0	25	-	-	1	4	5	5	7.2-6.5	6.5-5.8	6.9	6.1	0.8	Diarrhea after 15 gm

\*Two doses of 25 gm each



## SUMMARY AND CONCLUSIONS

Under controlled experimental conditions gluconic acid given in the form of the delta lactone in large doses reduces to low levels the pH of the urine in man. Its acidifying action under the ordinary conditions of diet and fluid intake, however, appears limited, since under these conditions smaller doses yielded equivocal results on the pH, and even these caused a high incidence of gastrointestinal disturbance. From the practical standpoint, further experimentation is necessary to establish the conditions under which the delta lactone of gluconic acid may prove useful as a urinary acidifying agent.

## REFERENCES

1. Salkowski, E.: Ueber das Vorkommen von Pentosen im Harn, *Ztschr. f. physiol. Chem.* 27: 507, 1899.
2. Mayer, P.: Experimentelle Untersuchungen über Kohlenhydratsäuren, *Ztschr. f. klin. Med.* 47: 68, 1902.
3. Baumgarten, O.: Ein Beitrag zur Kenntnis des Diabetes mellitus, *Ztschr. f. exper. Path. u. Therap.* 2: 53, 1905.
4. Hermann, S.: Zur Pharmakologie der Glucensäure, *Arch. f. exper. Path. u. Pharmacol.* 154: 175, 1930; *Ibid.* 154: 143, 1930.
5. Schott, E.: Über Verhalten von Glyconsäure und Zuckersäure im Organismus, *Arch. f. exper. Path. u. Pharmacol.* 65: 35, 1911.
6. Schwarz, L.: Untersuchungen über Diabetes, *Deutsches Arch. f. klin. Med.* 76: 233, 1903.
7. Baer, J., and Blum, L.: Über die Einwirkung chemischer Substanzen auf die Zuckerausscheidung und die Acidose, *Arch. f. exper. Path. u. Pharmacol.* 65: 1, 1911.
8. Paderi, C.: Sur la mode de se comporter de l'acide glyconique dans l'organisme (Abst.), *Zentralbl. f. Biochem. u. Biophys.* 19: 622, 1918.
9. Hermann, S.: Methode zur Auflösung von Phosphatkonkrementen der Harnwege, *München. med. Wchnschr.* 82: 540, 1935.
10. Sisk, I., and Toenhart, O.: Gluconic Acid as a Urinary Acidifying Agent, *J. Urol.* 39: 699, 1938.
11. Johnston, J. M.: A Review of the Pharmacology of Urinary Antiseptics, *Lancet* 1: 54, 1932.

## STAPHYLOCOCCUS EPIDERMIS ALBUS\*

## CULTURAL AND IMMUNOLOGIC REACTIONS OF LARGE AND SMALL COLONY TYPES

ESTHER MEYER, PH.G., M.S., CHICAGO, ILL.

IT HAS long been noted that upon the swabbing of skin surfaces and subsequent plating on agar, both large and small staphylococcus colonies appear. After a thorough washing of the skin with soap and water, the number of small colony staphylococci increases. Colonies measuring 1 mm. or less in diameter were classified as "small colony type," while those measuring from 2 mm. to 4 mm. were classified as "large colony type." Growth produced by the small colony type was always rather scanty and frequently failed to survive transfer from the original plates.

*Technique.*—The skin was thoroughly washed with soap and water and dried with sterile gauze. A sterile moist swab was used to swab the skin area.

\*From the Department of Bacteriology and Public Health, University of Illinois College of Medicine, and the Department of Bacteriology, University of Illinois College of Pharmacy, Chicago.

It was then thoroughly rubbed over an agar plate. The plate was incubated for forty eight hours. Only albus colored colonies of both sizes were selected for study. Subcultures were made on agar plates to insure purity. After a forty-eight hour incubation period, 25 typical colonies measuring 2 mm. to 4 mm., and 25 colonies, measuring 1 mm. or less, were picked and transferred to agar slants.

TABLE I  
STAPHYLOCOCCUS EPIDERMIS ALBUS (LARGE COLONY TYPES)  
Isolated From the Surface of the Skin

STRAIN	TITANUS MILK	NITRATE	CELESTIN	DEXTROSE	MANNITE	MALTOSF	LACTOSF	RAFFINOSF	SUCROSE	INULIN	CUM STAIN
1	+	+	+	+		+	+		+		+
2	+	+	+	+	+	+			+		+
3	+	+	+	+		+			+		+
4	+	+	+	+		+			+		+
5	+	+	+	+		+			+		+
6	+	+	+	+		+			+		+
7	+	+	+	+		+	+		+		+
8	+	+	+	+		+			+		+
9	+	+	+	+		+			+		+
10	+	+	+	+		+			+		+

TABLE II  
STAPHYLOCOCCUS EPIDERMIS ALBUS (SMALL COLONY TYPES)  
Isolated From the Surface of the Skin

STRAIN	TITANUS MILK	NITRATE	CELESTIN	DEXTROSE	MANNITE	MALTOSF	LACTOSF	RAFFINOSF	SUCROSE	INULIN	CUM STAIN
1	+	+	+	+	+	+			+		+
2	+	+	+	+	+	+			+		+
3	+	+	+	+	+	+	+		+		+
4	+	+	+	+	+	+	+		+		+
5	+	+	+	+	+	+			+		+
6	+	+	+	+	+	+			+		+
7	+	+	+	+	+	+		+	+		+
8	+	+	+	+	+	+			+		+
9	+	+	+	+	+	+		+	+		+
10	+	+	+	+	+	+			+		+

TABLE III  
STAPHYLOCOCCUS EPIDERMIS ALBUS (LARGE COLONY TYPES)  
Biochemical Groups

GROUP	NUMBER OF STRAINS	TITANUS MILK	NITRATE	CELESTIN	DEXTROSE	MANNITE	MALTOSF	LACTOSF	RAFFINOSF	SUCROSE	INULIN
1	8 (44)*	+	+	+	+		+	+		+	
2	2 (50)*				+	+		+		+	
3	1 (41)*		+	+	+		+			+	
4			+		+		+	+		+	

\*Strains used for immunologic experiments

TABLE IV  
STAPHYLOCOCCUS EPIDERMIS ALBUS (SMALL COLONY TYPES)  
Biochemical Groups

GROUP	NUMBER OF STRAINS	LITMUS MILK	NITRATE	GELATIN	DEXTROSE	MANNITE	MALTOSE	LACTOSE	RAFFINOSE	SACCHAROSE	INULIN
1	5	+	+	+	+	-	+	+	-	+	-
2	6 (11s) <sup>*</sup>	+	-	+	+	-	+	+	-	+	-
3	6 (22s)	-	+	+	+	-	+	-	-	+	-
4	8 (18s) <sup>*</sup>	-	+	-	+	+	+	-	-	+	-

\*Strains used for immunologic experiments

TABLE V

	LACTOSE	MALTOSE	DEXTROSE	LITMUS MILK	PER CENT
Large staphylococci colonies (skin)	+	+	+	+	28
Large staphylococci colonies (skin)	-	+	+	-	20
Large staphylococci colonies (skin)	-	-	+	-	9
Large staphylococci colonies (skin)	+	+	+	-	36
Large staphylococci colonies (skin)	-	+	+	+	8

TABLE VI

	LACTOSE	MALTOSE	DEXTROSE	LITMUS MILK	PER CENT
Small staphylococci colonies (skin)	+	+	+	+	22
Small staphylococci colonies (skin)	-	+	+	-	50
Small staphylococci colonies (skin)	-	-	+	-	18
Small staphylococci colonies (skin)	+	+	+	-	9

TABLE VII

STAPHYLOCOCCUS EPIDERMIS ALBUS (SKIN)	REDUCTION OF NITRATES		LIQUEFACTION OF GELATIN		MANNAN MANNITE	
	Per Cent		Per Cent		Per Cent	
	Positive	Negative	Positive	Negative	Positive	Negative
Large colony type	64	36	52	48	28	72
Small colony type	68	32	64	36	22	78

TABLE VIII

AGGLUTINATION REACTIONS  
Large and Small Staphylococci Colonies

	SERUM 44	SERUM 50	SERUM 18s	SERUM 11s	SERUM 22s
Strain 44	640	2560	320	320	160
Strain 50	40	640	0	40	20
Strain 44*	2560	40	320	80	320
Strain 18s	320	2560	640	640	0
Strain 11s	320	20	0	40	80
Strain 22s	20	80	80	+	

Numbers 11s, 18s, and 22s are small colony types

Numbers 44, 50, and 44 are large colony types

\*Rabbit died five days after last injection hence no serum was obtained

†Rabbit died five days after trial bleeding Not enough serum remained to run test

In this work we were interested in determining whether there are any marked differences in cultural and immunologic reactions between the large and small colony staphylococci found on the skin.

*Biochemical Reactions*—Subcultures were made in litmus milk, nitrate peptone solution, gelatin, dextrose, mannite maltose, lactose raffinose, sucrose, and milk. Table I shows the cultural reactions of the first ten strains in the large colony group, and Table II shows those of the first ten in the small colony group. The cultures were grouped according to biochemical reactions and Tables III and IV show the number belonging to each group. Throughout the work it was evident, as is shown in the tables, that the small colony types are not as active biochemically as the large colony types. Tables V and VI show that 64 per cent of the large type ferment lactose as compared with only 31.5 per cent of the small type. Table VII shows the small type to be more active gelatin liquefiers than the large type.

*Immunologic Reactions*—Three strains were selected from the large colony types (No. 44 from group 1, No. 50 from group 2 and No. 41 from group 3), and three strains from the small colony types (No. 11s from group 2, No. 22s from group 3, and No. 18s from group 4). Rabbits were selected for the immunologic work. Heavy saline suspensions of the living organisms were injected intravenously, three injections being given in all. Ten days after the last injection, trial bleedings were made and the titers of sera tested. The rabbits were then bled to completion. During the course of the work one death occurred (No. 41) five days after the last injection. The cause could not be determined. Another rabbit (No. 11s) died following the trial bleeding. No serum was obtained from rabbit No. 41 but enough was obtained from No. 11s in the trial bleeding to suffice for all but one test. The rabbits showed more reaction after injection with the large type colony staphylococci than the small type colony staphylococci.

The agglutination reactions are summarized in Table VIII. It is evident from these results that all of these strains are related to each other. The large colony type have broader antigenic relationships hence they may be considered the parent strain. Strain 50 is type specific while strains 44, 41, and 18s have wide antigenic relationships. The agglutinins in antisera 44 and 50 have a wide band of activity.

#### SUMMARY

The results of this work indicate

- (1) All of these strains are related to each other.
- (2) The large colony types are more highly antigenic, have wider antigenic relationships and therefore may be considered the parent strain.
- (3) The large colony types are more active biochemically than the small types.
- (4) The growth produced by the small colony type is always limited as compared to that of the large colony type.

#### REFERENCES

- Arnold, L., Gustafson, C. J., Hull, T. G., Montgomery, B. E., and Singer, Charlotte. The Self Disinfecting Power of the Skin as a Defense Against Microbic Invasion, *Am. J. Hyg.* 11: 345, 1930.

- Colebrook, L., and Moxted, W. R. *Antisepsis in Midwifery*, J. Obst. & Gynaec Brit Emp 40: 966, 1933.
- Cowan, S. T.: The Classification of Staphylococci by Precipitation and Biological Reactions, J. Path. & Bact. 46: 31, 1938.
- Hine, T. G. M.: Serological Classification of the Staphylococci, Lancet 2: 1380, 1922.
- Juhanelle, L. A.: Studies of Hemolytic Staphylococci Agglutinations, J. Infect Dis 31: 256, 1922.
- Juhanelle, L. A., and Wiegard, C. W.: Immunological Specificity of Carbohydrate Derived From Staphylococci, Proc. Soc. Exper. Biol. & Med 31: 947, 1933.
- Juhanelle, L. A., and Wiegard, C. W.: The Immunological Specificity of Staphylococci II The Chemical Nature of the Soluble Specific Substances, J. Exper. Med 62: 31, 1935.
- Klopstock and Bochenheimer: Beitrag zur Agglutination der Staphylokokken, Arch f Klin Chir 72: 325, 1904.
- Koch, Joseph: Ueber das Vorkommen Pathogener Staphylokokken auf der Korperoberflaeche des Menschen und Seiner Umgebung, Ztschr. f. Hyg. u Infektionskr 58: 257, 1908.
- Kutscher and Komich: Untersuchungen ueber die Beziehungen von Haemolysinbildung und Agglutinabilitat der Staphylokokken, Ztschr. f. Hyg. u Infektionskr. 48: 249, 1904.
- Montgomery, B., and Gustafson, C. J.: Classification of Staphylococcus Epidermis Albus From Human Skin, Proc. Soc. Exper. Biol. & Med. 27: 365, 1930.
- Winslow, C. E. A., Rothberg, Wm., and Parsons, Eliz. L.: Notes on the Classification of the White and Orange Staphylococci, J. Bact. 5: 145, 1920.
- Beigev, D. H.: Manual of Determinative Bacteriology, Baltimore, 1939, Williams & Wilkins Co.

## A STUDY OF ENZYMES IN NORMAL AND PATHOLOGIC CEREBROSPINAL FLUID<sup>1</sup>

I KAPLAN, PH.D, D. J. COHN, PH.D, A. LEVINSON, M.D,  
AND B. STERN, B.S, CHICAGO, ILL.

**A** KNOWLEDGE of the enzymes of the cerebrospinal fluid may lead to a clearer understanding of the physiology of the central nervous system and may also be of clinical value in diseases of the central nervous system. It is not surprising, therefore, that the study of the enzymes of the cerebrospinal fluid has attracted a number of workers.<sup>1,2</sup> As yet, however, no adequate correlation has been made between the occurrence of enzymes in the cerebrospinal fluid and the condition of the central nervous system.<sup>2</sup> Some workers have limited themselves to the study of only one or two enzymes,<sup>3,4</sup> or to only a few pathologic conditions, such as mental diseases.<sup>5</sup> Others have studied the factors which regulate the permeability of the meninges<sup>6,7</sup> and have not been interested in the enzymes of the cerebrospinal fluid.

In an attempt to investigate the relationship between the condition of the central nervous system and the distribution of enzymes in the cerebrospinal fluid, we studied the enzymatic activity of over one hundred specimens of normal and pathologic cerebrospinal fluid. The enzymes studied were trypsin,<sup>†</sup> phosphatase, lipase, tributyrinase, esterase, and amylase. Anti-

<sup>1</sup>From the Department of Biochemistry, Nelson Morris Institute for Medical Research the Children's Hospital of the Cook County Hospital, and the Sarah Morris Hospital for Children of the Michael Reese Hospital.

Aided by a grant from the Nelson Morris Fund.

Received for publication, January 26, 1939.

<sup>†</sup>Throughout the paper certain conventions will be used with respect to the nomenclature of the enzymes. By trypsin is meant the enzyme system which catalyzes the breakdown of denatured hemoglobin in a slightly alkaline medium. The identity of the enzyme with pancreatic trypsin or other tryptic enzymes is not discussed here. The term 'trypsin' has been chosen for convenience. Similarly, by lipase is meant the enzyme system which hydrolyzes olive oil, by tributyrinase, the enzyme which hydrolyzes tributyrin, by esterase, the enzyme which hydrolyzes ethyl butyrate, etc.

trypsin, although not an enzyme was studied in connection with trypsin. Cell counts were done in each instance and determinations of the sugar, chlorides, total protein and nonprotein nitrogen contents of the fluids were made when ever possible\*. When spinal punctures were necessary for diagnostic or therapeutic purposes specimens of cerebrospinal fluid were obtained from hospital patients, most of whom were children. Most of the punctures were lumbar, when ventricular or cisternal punctures were resorted to they are so marked in the tables. The fluids were withdrawn after an overnight fast, and enzymatic and chemical studies were begun within one or two hours. Unless otherwise indicated, the fluids were clear and colorless.

#### METHODS

Trypsin was determined by the method of Anson and Minsky<sup>10</sup> with modifications made necessary by the nature of the material analyzed. In this method the amount of tyrosine formed as a result of the digestion of a hemoglobin substrate by a tryptic enzyme is measured colorimetrically. The method as modified for the purpose of this work is here presented.

One cubic centimeter of cerebrospinal fluid was added to 2 cc of hemoglobin substrate and the mixture was incubated at 37° C. for twenty-four hours. An incubation period of four hours is sufficient to yield results, but the longer incubation period gives greater precision because of the larger amounts of tyrosine formed. Ten cubic centimeters of 5 per cent trichloroacetic acid were then added to the digestion mixture to precipitate the undigested hemoglobin and to stop the enzyme reaction. After five minutes the solution was filtered. To 5 cc of filtrate were added 10 cc of 0.5 N sodium hydroxide and 3 cc of phenol reagent diluted 1:3. The color which developed was compared in the colorimeter with a suitable tyrosine standard set at 20. The standards were prepared by adding to 5 cc of tyrosine solution containing 0.10, 0.15, or 0.25 mg of tyrosine 10 cc of 0.5 N sodium hydroxide and 3 cc of diluted phenol reagent. A blank, or zero time determination was run simultaneously by adding 1 cc of cerebrospinal fluid to 5 cc of substrate solution at 37° C., and adding immediately 10 cc of 5 per cent trichloroacetic acid. The difference between the number of millmoles of tyrosine found after incubation and without incubation was taken as the measure of the tryptic activity of the cerebrospinal fluid. Calculations of the tryptic activity were made, using modifications of the methods of calculation of Anson and Minsky similar to those of Hecht and Crim.<sup>11</sup> These modifications were necessary because of the wide variation of the amounts of trypsin found in cerebrospinal fluid, requiring the use of more than one standard. The number of millmoles of tyrosine found colorimetrically can be written<sup>11, 12</sup>

$$(1) \text{ mM Tyrosine} = \frac{16}{5} \times \frac{1}{181} \times \frac{20}{\lambda} (\text{mg tyrosine standard}) - \frac{\text{reagent}}{\text{blank}}$$

The number of trypsin units per 100 cc of cerebrospinal fluid is therefore,

$$(2) \text{ mM Tyrosine after 24 hours} - \text{mM tyrosine at 0 hours} = \text{TU per 100 cc} = \frac{1600}{905} \left[ \frac{20}{\lambda} (\text{mg T}_{\text{St}}^{24}) - \frac{20}{\lambda} (\text{mg T}_{\text{St}}^0) \right]$$

\*Studies were also made of the calcium and phosphorus contents. These have been reported elsewhere (J. LAB. & CLIN. MED. 24: 609, 1939).

where  $\text{mg. } T \frac{24}{\text{St.}}$  = the number of milligrams of tyrosine in the standard required for the twenty-four-hour reading, and  $\text{mg. } T \frac{0}{\text{St.}}$  is the number of milligrams of tyrosine in the standard required for the initial reading.  $x_{24}$  and  $x_0$  are the respective colorimetric readings. For example, if the 0.25 mg. tyrosine standard is required at twenty-four hours, and 0.10 mg. standard for the initial reading, the number of trypsin units per 100 c.e. is:

$$(3) \text{ T.U.} = 1.77 \left[ \frac{20}{x_{24}} (0.25) - \frac{20}{x_0} (0.10) \right] \\ = 1.77 \left[ \frac{5.0}{x_{24}} - \frac{2.0}{x_0} \right]$$

Antitrypsin was determined by measuring the effect of the presence of cerebrospinal fluid on the activity of a commercial trypsin preparation. To 5 e.e. of hemoglobin substrate were added 1 e.e. of a solution (1:1000) of commercial trypsin and 1 e.e. of water or of cerebrospinal fluid. The percentage inhibition of the particular trypsin used was taken as the measure of the antitryptic power of the cerebrospinal fluid. The decrease in millimoles of tyrosine formed in the presence of cerebrospinal fluid is given by the equation:

$$(4) \text{ mM. Tyrosine} = \frac{1700}{905} \left[ \frac{20}{x_{\text{H}_2\text{O}}} (\text{mg. } T \frac{\text{H}_2\text{O}}{\text{St.}}) - \frac{20}{x_{\text{CSF}}} (\text{mg. } T \frac{\text{CSF}}{\text{St.}}) \right],$$

where  $\text{mg. } T \frac{\text{H}_2\text{O}}{\text{St.}}$  and  $\text{mg. } T \frac{\text{CSF}}{\text{St.}}$  are the numbers of milligrams of tyrosine in the standards required when water and cerebrospinal fluid, respectively, were added to the hydrolysis mixture;  $x_{\text{H}_2\text{O}}$  and  $x_{\text{CSF}}$  are the corresponding colorimetric readings. The number 1700 appears in the formula instead of 1600 because the digestion mixture on addition of 5 per cent trichloroacetic acid contains 17 e.e. instead of 16 e.e. as in the determination of trypsin. The percentage decrease in millimoles of tyrosine formed is then:

$$(5) \text{ Antitrypsin units} = \text{per cent inhibition} = \\ \frac{\frac{20}{x_{\text{H}_2\text{O}}} (\text{mg. } T \frac{\text{H}_2\text{O}}{\text{St.}}) - \frac{20}{x_{\text{CSF}}} (\text{mg. } T \frac{\text{CSF}}{\text{St.}})}{\frac{20}{x_{\text{H}_2\text{O}}} (\text{mg. } T \frac{\text{H}_2\text{O}}{\text{St.}}) - \text{reagent blank}} \times 100.$$

The phosphatase activity of the cerebrospinal fluid was determined by the method of Bodansky,<sup>12</sup> which depends on the determination of the inorganic phosphorus liberated from a phosphoric acid ester by the action of the enzymatic material. Because of the small phosphatase activity usually found in the cerebrospinal fluid, a few slight modifications of the above-mentioned method were necessary. Ten cubic centimeters of buffered substrate, sodium-beta-glycerophosphate and monosodium diethylbarbiturate, and 2 e.e. of cerebrospinal fluid were incubated at 37° C. for twenty-four hours in a small rubber-stoppered flask. At the end of this time the flask was cooled, 8 e.e. of 5 per cent trichloroacetic acid were added, and the mixture was shaken and filtered. To 5 e.e.

of this filtrate were added 4 cc of molybdate reagent and 1 cc of stannous chloride solution. The blue color developed was read in a colorimeter against the color developed on similar treatment with molybdate and stannous chloride by a standard phosphate solution containing 0.004 mg per cc. The difference in milligrams of phosphorus found after incubation and that initially present in the cerebrospinal fluid was taken as the measure of phosphatase activity.

Lipase was determined by a slight modification of the method of Crandall and Cherry,<sup>13</sup> which depends on the determination by titration with sodium hydroxide of the free acid formed by the enzymatic hydrolysis of olive oil. A mixture containing 3 cc of distilled water, 2 cc of a 50 per cent olive oil emulsion made up with 5 per cent gum acacia as the emulsifying agent, 0.5 cc of Clark and Lubs' buffer at pH 7.0 and 1 cc of cerebrospinal fluid was incubated at 37° C for twenty-four hours and then titrated with N/20 sodium hydroxide. Since it was found that some hydrolysis of the olive oil took place even though no cerebrospinal fluid was present in the above mixture a blank was run using 1 cc of water instead of cerebrospinal fluid. The lipolytic activity, expressed in millimoles of fatty acid formed in twenty-four hours per 100 cc of cerebrospinal fluid is

$$\text{Lipase units} = (\text{cc NaOH}_{\text{test}} - \text{cc NaOH}_{\text{blank}}) \times 5 \times 100$$

The tributyrin and ethyl butyrate splitting power of the cerebrospinal fluid was determined by the above method replacing the 2 cc of olive oil emulsion with 1 cc of absolute tributyrin or 1 cc of absolute ethyl butyrate. The calculations for tributyrinase and esterase were the same as those for lipase.

The amylolytic activity of the cerebrospinal fluid was determined by a modification of the method of Ross and Shaw<sup>14</sup> which depends on the hydrolysis of starch or glycogen by the enzymatic material and the oxidation of the resulting reducing sugars (considered as maltose) by hypiodite. To a mixture of 25 cc of freshly prepared 1 per cent starch solution, 10 cc of phosphate buffer at pH 6.8, 1 cc of 0.2 N sodium chloride, and 3 cc of distilled water, was added 1 cc of cerebrospinal fluid. The resulting mixture was shaken and 10 cc were pipetted into a small Erlenmeyer flask containing 0.5 cc of N hydrochloric acid. The acid and phosphate buffer were neutralized with N sodium hydroxide. Then 2 cc of 0.1 N iodine solution and 3 cc of 0.1 N sodium hydroxide were added and the mixture allowed to stand for fifteen minutes. One cubic centimeter of N hydrochloric acid was added to liberate the excess iodine, which was titrated with 0.02 N sodium thiosulfate. After twenty-four hours, 10 cc of the hydrolysis mixture were withdrawn and treated similarly. The measure of amylolytic activity was taken as

$$\begin{aligned} \text{Amylase units} &= \text{millimoles of maltose formed in 24 hours per 100 cc of cerebrospinal fluid} \\ &= \frac{(\text{N}_2\text{S}_2\text{O}_4 \text{ titer in cc after 0 hours} - \text{N}_2\text{S}_2\text{O}_4 \text{ titer in cc after 24 hours})}{\times 4 \times 100} \end{aligned}$$

Since 1 cc of 0.02 N  $\text{N}_2\text{S}_2\text{O}_4$  = 3.41 mg malto c = 0.01 millimoles maltose



TABLE I

## NORMAL CEREBROSPINAL FLUID

CASE	DIAGNOSIS	CELL COUNT	TRYPSIN <i>mM.</i>	ANTI-TRYPSIN UNITS	PHOS- PHATASE <i>Mg.</i> <i>Phosphorus</i>	LIPASE		THIRI- TRYPSINASE	ESTERASE		AMYLASE <i>mM.</i> <i>Maltose</i>	TOTAL PROTEIN	SUGAR	CHLO- RIDES	NON- PROTEIN NITROGEN
						<i>mM.</i> <i>Fatty Acid</i>	<i>mM.</i> <i>Fatty Acid</i>		<i>mM.</i> <i>Fatty Acid</i>	<i>mM.</i> <i>Fatty Acid</i>					
B. B.	Epilepsy	1	0	0	0.4	0	0	0	0	0	0.8	19	54	730	13
R. P.	Epilepsy	3	0	0	0	0.4	0	0	0	0	2.0	14	58	712	14
J. B.	Epilepsy	4	0	0	0	0	0	0	0	0	0.6	25	58	687	14
W. S.	Epilepsy	3	0	0	0.9	0.8	0.8	0.8	0	0	0	7	75	735	
J. A.	Epilepsy	3	0	0	0	0	0	0	0	0	1.0	3	50	715	20
L. N.	Epilepsy	6	0	0	0.4	0.9	0	0	0	0	1.2	46	56	748	14
L. B.	Epilepsy	0	0	0	0	0	0	0	0	0	2.0	5	45	710	12
N. W.	Epilepsy	0	0	0	0	0	0	0	0	0	1.8	13	47	718	17
M. M.	Cerebral birth trauma	8	0	8	0.2	0.3	0	0	0	0	0	17	67	731	
D. Ma.	Cerebral birth trauma	12	0	0	0.3	0	0	0	0	0	0				
J. S. (1)	Cerebral birth trauma	0	0	0	0	0	0	0	0	0	0				
J. S. (2)	Cerebral birth trauma	7	0	9	1.8	0	0	0	0	0	0.6	8	53	702	18
C. H. (1)	Cerebral birth trauma	2	0	20	0	0	0	0	0	0	3.3				
O. H. (2)	Cerebral birth trauma	3	0	0	0	0.8	0	0	0	0	0	31		721	17
A. F. (1)	Cerebral birth trauma	4	0	0	0	0	0	0	0	0	1.0	31			8
A. F. (2)	Cerebral birth trauma	2	0	0	0	0	0	0	0	0	0	13			10
N. J.	Cerebral birth trauma	4	0	0	1.6	0	0	0	0	0	0.7	26	50	702	10
M. K. (1)	agenesis Cerebral	7	0	20	0	0	0	0	0	0	0.8		61		
M. K. (2)	agenesis Cerebral	0	0	0	1.4	0	0	0	0	0	0				
J. Be.	agenesis Cerebral	5	0	0	0	0	0	0	0	0	1.7	Pandy -	62	729	17
S. S.	Idiocy	4	0	0	0	0	0	0	0	0	2.8	28	45	710	18
F. L.	Idiocy	3	0	0	1.8	0	0	0	0	0	1.0				
D. R.	Idiocy	6	0	0	1.9	0	0	0	0	0	0			730	
M. F.	Idiocy	0	0	0	0	0	0	0	0	0	0				18
H. A. F.	Idiocy	0	0	0	0	0	0	0	0	0	0				

Glycogenase was determined by the same method as amylase, except that 0.5 per cent glycogen was used as a substrate instead of starch. In this case it was necessary to use 3 drops of 1 per cent starch solution as an indicator for the thiosulfate titration.

Sugar was determined by the method of Somogyi,<sup>17</sup> chlorides by the method of Van Slyke,<sup>18</sup> total protein by the method of Aver, Dailey and Fremont Smith,<sup>19</sup> nonprotein nitrogen by the method of Koch and McMeekin.<sup>20</sup> Total proteins were also done by a digestion method which was checked against the above mentioned method for total protein.

## RESULTS

For convenience in describing and discussing our results, we have divided the fluids studied into several groups.

*Group I: Normal*—The fact that a spinal puncture is considered necessary indicates that the patient is not entirely normal, as has been pointed out previously.<sup>1</sup> However, as can be seen from Tables I and VII, the cell counts and sugar, chlorides, total protein, and nonprotein nitrogen contents of these fluids were normal.<sup>1, 9, 20, 22</sup> Though not reported in this paper, the calcium and phosphorus contents of these fluids were also found to be normal. The enzyme distribution in these specimens was therefore considered to be that of normal cerebrospinal fluid.

None of the 27 fluids of the normal group showed any evidence of tryptic activity. Of 22 fluids studied 18 had no antitryptic properties; the remaining 4 fluids had slight antitryptic power. Phosphatase was found in 12 of 13 fluids, with an average activity of 0.9 unit (see methods for explanation of the units used throughout the paper). The actual values of the phosphatase activity ranged from 0.2 to 1.9 units. Lipolytic activity was found in small amounts in 4 fluids of 24 studied. The average lipolytic activity of the 24 fluids was negligible, being within the experimental error. Tributyrinase was found in a small amount, 0.8 unit, in only 1 of 20 fluids studied. Esterase was not found in any of the 24 fluids studied. Amylase was present in 17 of 25 fluids of group I, the average amylolytic activity of the 25 fluids being 0.9 unit. The individual activities of the 17 fluids which gave positive results varied from 0.4 to 3.3 units. Discussion of glycogenase is omitted since in all cases where glycogenase activities were determined the results paralleled those of amylase.

It appears from these results that beta glycerophosphatase and amylase are present in detectable quantities in an appreciable number of cerebrospinal fluids with normal chemical and cellular findings. Trypsin and esterase were not found. Lipase, tributyrinase, and antitrypsin were found in small amounts in relatively small percentages of the fluids studied.

*Group II: Tuberculous Meningitis*—In this group there were 23 fluids obtained from 13 patients. In general, the cellular and chemical constituents were in accord with the literature. In one case there were a low cell count and also a normal chloride content. This was due to the fact that the fluid was drawn during the early stage of the meningitis which followed a tuberculoma. The same explanation covers the normal sugar content in another case.

TABLE II  
TUBERCULOUS MENINGITIS

CASE	APPEARANCE OF FLUID	CELL COUNT	TRYPSIN	ANTI-TRYPSIN UNITS	PHOSPHATASE	LIPASE	TRYPHOSINASE	ESTERASE	AMYLASE	TOTAL PROTEIN	SUGAR	CHLO-RIDES	NONPROTEIN NITROGEN
			mM. Tyrosine		Mg. Phosphorus		mM. Fatty Acid		mM. Maltose		Mg. Per cent		
C. W.	Clear	150	0.03	21		0	0	0	2.1	Pandy +	16	648	
*H. W. (1)	Clear	19	0.01	26		0	0.9	0	0	275	13	724	14
H. W. (2)	Clear	65	0.06	70		0	1.5	0	1.1	Pandy +			
I. W. (1)	Clear	220	0.17	0		0	0	0	1.4	Pandy +	27	679	
L. W. (2)	Pink (Piontosil)	160	0.12	16	1.8	0	0	0	1.8	Pandy +	24	700	
L. W. (3)	Pink (Piontosil)	175	0.11	45		0	0.7	0	1.0	Pandy +	20	700	
L. S. (1)	Clear	450	0.01	0	1.3					Pandy +	40		
L. S. (2)	Clear	450	0.23	6	2.9	0.5			0	Pandy +	36		
L. S. (3)	Clear	590	0.13	88	4.7	2.1	3.2			700	16		26
R. C.	Opalescent	350	0.01	19	1.1	1.1	1.3	0	1.6	67	26	722	
E. T.	Clear		0.04	0	0.9					Pandy +			
G. R. (1)	Clear	175	0.08	35	1.7	0	0.7	0	0	Pandy +	13	605	19
G. R. (2)	Opalescent	180	0.16	47	1.8	0.8	2.5	0		210	10		
G. R. (3)	Opalescent	850	0.30	80	2.2		0.8	0		174	10		
G. R. (4)	Opalescent	700	0.08	50	2.8	0.5	0.8	0	3.6	235	10		
C. F.	Clear	98	0.01	23	2.6	1.3	2.7	0	4.2	200	10	569	
R. N.	Turbid		0.29	0					2.8	171		656	
L. C.	Clear	190	0.08	19		0.5		0	2.8	Pandy +			
M. W. (1)	Opalescent	124	0.10	0		0		0	5.5	50		619	22
M. W. (2)	Opalescent		0.10	9		1.6		0	1.8	106			6
I. W. (1)	Clear	126	0	25	1.7	0	0.1	0	1.0	69	17	587	14
I. W. (2)	Clear		0.03	47	3.1	0	0.8	0	2.6	83	27	605	
I. R. M. P.	Clear	160	0	25	0.9	0	0	0	1.1	105	30	675	
										Pandy +	28		

\*Tuberculum of the brain followed by meningitis.

†Diagnosis unconfirmed.

TABLE III

## PULPIT MNINGITIS

CASE	DIAGNOSIS	APPEARANCE OF FLUID	CELL COUNT	PROTEIN	ANTI FLUKEIN UNITS	PHOSPHATASE	ALPASE	TYRINASE	TIBL	FESTINASE	AMYLASE	TOTAL PROTEIN	SUGAR	CHLO RIDES	NON PRO TEIN NITRO GEN
A McQ (1)	Influenzal	Turbid	5000	mM Tyrosine 0.08	91	Mg Phosphorus	0.7	mM Tatty Acid 2.0	0	mM Maltose 0	0	481	10	673	21
A McQ (2)	Influenzal	Turbid	7200	0.15	44		0	0	0	0	0		10		24
A McQ (3)	Influenzal	Turbid	11000	0.15	50		4	1.9	0.9	2.2	0		10		
A McQ (4)	Influenzal	Turbid	2000	1.20	8		4	0	0	1.6	0		10		
R R W	Influenzal	Cysternal turbid	5000	1.44	24		0	1.4	1.0	0	0		10	682	
R R H	Influenzal	Cysternal turbid		0.80	17		0	0	0	2.4	0	481	10	669	
W G (1)	Streptococci (in hemolyticus) and	Cysternal turbid	600	0.26	0	9.0	0	0	0	0	0	38	10		
W G (2)		Cysternal turbid	4600	1.40	0		0								
W G (3)	Streptococci (alpha)	Ventricular turbid	5000	1.80	0	22.0	1	3	2.9	2.8	0	942	18	732	
G O (1)	Streptococci (hemolytic and viridans)	Ventricular turbid	3400	0.71	4		0		0	0.8	0				
G O (2)		Ventricular turbid	720	0.18	64		0	0	0	2.6	0	Pandy ++++	10	650	
G O (3)	Streptococci (hemolytic and viridans)	Ventricular turbid	900	0.60	61		0	0	0	2.0	0	Pandy ++++			
J D (1)	Streptococci (hemolytic)	Ventricular turbid	2290	0.53	39		0	0	0	0	0	81			13
J D (2)	Streptococci (hemolytic)	Opalescent	440	0.20			0					32			18
J B	Streptococci	Turbid	6000	1.30	10	22.0	0								
J T	Pneumococci	Turbid	5000	1.70	66	14.0	0	1.7				375	27*	758*	
D R (1)	Meningococci	Turbid	3000	0.67	7	14.4	2.5	2.2	0.6	0	0	89	58*	736*	
D R (2)	Meningococci	Turbid	1500	0.40	0	8.0	1.3	3.4	0.9	0.8	0	61			

\*These values are of doubtful significance because of the large amounts of intravenous glucose and saline which the patient received during treatment.

On the whole the cerebrospinal fluid of tuberculous meningitis was characterized by increased enzymatic activity. Most striking was the presence of trypsin and of increased amounts of antitrypsin. Twenty-one of 23 fluids had definite tryptic activity, ranging from 0.03 to 0.30 unit, with an average of 0.10 unit. Antitrypsin was found to be present in 16 of 22 fluids studied, with values for the antitryptic power from 6 to 88 units, and an average of 28 units for all the fluids. Phosphatase activity, studied in 14 fluids of this group, was found to be about twice as great as that of the normal fluids. Values from 0.9 to 4.7 units were found, with an average value of 2.1 units. In 8 of 20 fluids studied for lipolytic activity, values as high as 2.4 units were encountered; the average activity was 0.3 unit. Tributyrinase was present in 12 of 16 fluids, with activities as high as 3.2 units and an average of 1.0 unit. Esterase was not found in any of the 18 fluids tested for this enzyme. Amylase was present in 14 of 17 fluids studied. The average activity was 1.9 units, with a maximum value of 5.5 units.

*Group III: Purulent Meningitis.*—The results of the chemical studies agreed with those of the literature. The total protein contents were elevated, with values as high as 940 mg. per cent. Sugars and chlorides were nearly always reduced. White cell counts were markedly elevated, with values from 440 to 11,000 per c. mm., and a predominance of polymorphonuclear leucocytes.

In general, the distribution of enzymes in the fluids of the purulent meningitides was similar to that in tuberculous meningitis, except that far larger amounts of trypsin and phosphatase were present in purulent meningitis. All the 18 fluids studied contained trypsin, in amounts ranging from 0.08 unit to 1.80 units, with an average value of 0.87 unit. Fourteen fluids studied showed antitryptic power, varying from 4 to 91 units, with an average value of 31 units. Phosphatase activity was measured in only 6 fluids, but these were consistently high in phosphatase activity, ranging in value from 8 to 22 units, with an average value of 15 units. With one exception no higher value for phosphatase activity was found in any of the fluids studied in the whole series. Lipase was present in 6 of 16 fluids studied; its presence appeared to depend on the severity of the disease. Tributyrinase was found in 7 of 12 fluids, with activities as high as 3.4 units and an average of 1.3 units. Esterase appeared in some fluids in the advance stages of the disease. Amylase was present in 8 of 14 fluid studies, with an average value of 1.1 units.

*Group IV: Hydrocephalus.*—Nearly all of the fluids of this group presented a different enzyme picture from that of the normal fluids. The extent of the difference depended on the cause of the hydrocephalus and in some cases on the presence of infection. For convenience we shall distinguish between two subgroups of fluids. The first subgroup consisted of five fluids all of which were abnormal in appearance, being opalescent, xanthochromic, or turbid. In one patient, G. C., the hydrocephalus was found on autopsy to have been caused by occlusion of the foramen of Monro by an old hemorrhage into the third ventricle. In another patient, W. W., the hydrocephalus was

caused by a cerebellar medulloblastoma which invaded the fourth ventricle. The other three fluids of this subgroup were from two patients with hydrocephalus complicated by infection and showed definite leucocytosis. The protein contents of the 5 fluids were high ranging in value from 456 to 2500 mg per cent.

The sugar content of the infected fluids was zero; all of the noninfected fluids were normal. The chloride values were not significantly diminished. All the fluids had marked antitryptic power and were high in lipase, tributyrinase, and phosphatase. One fluid that of R. K. had a very high tryptic activity, two fluids with a relatively low polymorphonuclear content had low trypsin values, while the noninfected fluids were normal with respect to trypsin. Amylase values were normal and only one fluid that of R. K., showed esterase activity.

The fluids of the second subgroup, 13 in number, had relatively lower protein contents, the values ranging from 5 to 103 per cent. Eleven of the fluids were clear, one was contaminated by a few red cells and one fluid was xanthochromic. Seven fluids had tributyrinase activity with values from 0.7 to 3.0 units. Ten fluids had lipolytic activity with values from 0.6 to 7.7 units. With respect to the other enzymes and antitrypsin the fluids of this subgroup showed no significant variations from the normal.

On the whole, therefore, fluids from patients with hydrocephalus where there was an associated lesion invading a ventricle or which were complicated by infection, had greater enzymatic activity than fluids from patients in whom there was no known involvement of meninges or ventricles.

*Group V: Brain Tumor, Abscess, Cyst*—The fluids of this group were similar to those of Group IV in that they could be divided conveniently into two subgroups with greater or less enzymatic activity according to whether or not there was a lesion invading a ventricle or the meninges. The first subgroup includes one fluid from a patient with a medulloblastoma of the cerebellum invading the fourth ventricle (Table V, W. W.), four fluids from a patient with a cerebral subarachnoid cyst (R. McC.), and one fluid from a patient with an abscess of the right parietal lobe (C. K.). In the last case the abscess broke into the subarachnoid space so that the fluid was turbid, like that of a suppurative meningitis. The three cases of this subgroup have in common the fact that the lesions of the brain invaded spaces containing cerebrospinal fluid, viz., fourth ventricle and subarachnoid space. Of the five fluids making up the second subgroup, 2 were from a patient (E. P.) with a glioblastoma of the middle fossa, two from a patient (L. C.) with a cerebral abscess of undetermined location, and one from a patient with an intracerebellar glioma.

Of the 6 fluids of the first subgroup, 5 (W. W. and R. McC.) were xanthochromic and had highly elevated protein contents (416 to 660 mg per cent), the sixth (C. K.) had a total protein content of 103 mg per cent. The fluids of the second subgroup were clear, with protein contents ranging from 58 to 207 per cent. The average values of the chloride and sugar contents for all the fluids of group V fell within the ranges of normal values, with no significant individual variations.

TABLE IV  
HYDROCEPHALUS

CASE	APPEARANCE OF FLUID	CELL COUNT	TRYPSIN	ANTI-TRYPSIN UNITS	PHOSPHATASE	LIPASE	TRIBUTYRINASE		ESTERASE	AMYLASE	TOTAL PROTEIN	SUGAR	CHLORIDES	N.P.N.	COMMENT
			mM. Tyrosine		Mg. Phosphorus		mM. Fatty Acid			mM. Maltose	Mg. Per Cent				
First Subgroup															
W. H. (1)	Opalescent, ventricular	310 (Poly.)	0.04	65	6.9	1.0	2.1	0	0	680	0	646	-		Staph. aureus pyocephalus, spina bifida
W. H. (2)	Opalescent, ventricular	350 (Poly.)	0.03	34	14.2	1.1	4.3		1.7	650	0	69.4	0		
R. K.	Turbid, ventricular	Frank pus	2.32	26	66.4	1.1	7.4	3.9	0	2500	0				B. pyocyaneus pyocephalus, spina bifida
G. C.	Xanthochronic, ventricular		0	83		4.3		0	1.0	456			15		Occlusion of foramen of Munro by hemorrhage into choroid plexus
W. W.	Xanthochronic, lumbar	40 Lymph.	0	93	5.8	0.5	2.5	0	0.7	613	33	686	20		Medulloblastoma of cerebellum with invasion of the fourth ventricle





TABLE V  
BRAIN TUMOR, BRAIN ABSCESS, BRAIN CYST

BRAIN TUMOR, BRAIN ABSCESS, BRAIN CYST

CASE	DIAGNOSIS	APPEARANCE OF FLUID	CELL COUNT	TRYPSIN	ANTI TRYPSIN UNITS	PHOSPHATASE	LIPASE	TRIBUTYRINASE	ISOPERASE	AMYLASE	TOTAL PROTEIN	SUGAR	CHOL-ERIDES	NON-PROTEIN NITROGEN			
															Mg. Per cent		
															mM. Tyrosine	mM. Phosphorus	mM. Fatty Acid
First Subgroup																	
W. W.	Cerebellar medullo-blastoma	Xantho-chromic	40 Lymphs.	0	93	3.8	0.5	2.5	0	0.7	613	33	686	20			
R. McC. (1)	Cerebral subarach-noid cyst	Xantho-chromic	0	0	31		1.7		0	3.8	463	49	711	14			
R. McC. (2)	Cerebral subarach-noid cyst	Xantho-chromic	110 Lymphs.	0.18	69		0	1.3	0.6	0.9	416		722	15			
R. McC. (3)	Cerebral subarach-noid cyst	Xantho-chromic		0	28	2.0	3.0	2.8		1.0							
R. McC. (4)	Cerebral subarach-noid cyst	Xantho-chromic		0	88	7.7	0	3.0	0.5	1.8	660	53	814	15			
C. K.	Cerebral abscess	Turbid	2000 Polys.	1.40	38	11.4	1.0	5.8		7.8	103	56	710				
Second Subgroup																	
E. P. (1)	Glioblas-toma of middle fossa	Clear	120 Lymphs.	0	0	0.8	0.1	1.8			207						
E. P. (2)	Glioblas-toma of middle fossa	Clear	150 Lymphs.	0	0	1.0	1.2	1.8	0	0	154	29	685				
L. C. (1)	Cerebral ab-scess	Clear	170 Lymphs.	0.15	7	1.0	1.0	0.1			58						
L. C. (2)	Cerebral ab-scess	Clear	22 Lymphs.	0.01	18	0.7	0	0.7			80	63					
J. M.	Cerebellar glioma	Clear (ventricu-lar)	3 Lymphs.	0	0	0.8	5.1	2.3	0	0	13	93	800				

The fluids of the first subgroup possessed marked enzymatic properties. Two fluids of the 6 had high tryptic activities 0.47 and 1.4 units, respectively. All 6 fluids possessed antitryptic power with values ranging from 28 to 93 units and an average value of 58 units. Two fluids of this subgroup (W. W. and C. K.), examined for phosphatase activity, had values of 5.8 and 11.4 units, respectively. Four of the 6 fluids had lipolytic activity, with values from 0.5 to 3.0 units, and an average of 1.0 unit for the whole subgroup. Five fluids examined for tributyrinase activity gave positive results, with values from 1.3 to 5.8 units, and an average of 3.1 units. Of 4 fluids examined for esterase activity, 2 were found to have activities of 0.6 and 0.5 units, the other 2 had no esterase activity. The amylolytic activity of 6 fluids ranged from 0.7 to 7.5 units. The last value, obtained in patient C. K. was the highest amylase activity found in the entire study.

Of the second subgroup, 3 fluids (D. P. and J. M.) were similar to normal fluids with respect to trypsin, antitrypsin, phosphatase, esterase and amylase. All 3 fluids, however, had lipolytic activity 0.4, 1.2 and 5.4 units and tributyrinase activity (3.8, 1.8, and 2.3 units). The other 2 fluids (L. C.) had some tryptic activity (0.15 and 0.04 unit), some antitryptic power (7 and 18 units), and slight lipase and tributyrinase activities. The phosphatase was normal. On the whole, the fluids of this subgroup differed considerably less from the normal than those of the first subgroup.

**Group VI Miscellaneous.**—This group contains all fluids studied which did not fall into any of the five groups so far discussed. It includes fluids from patients with acute benign lymphocytic meningitis (2), poliomyelitis (3), and encephalitis (4), as well as one patient with cerebral arteriosclerosis, one patient with hemihypertrophy of the body, 3 patients with chorea, one patient with suspected carbon monoxide poisoning, one patient with postencephalitic syndrome, one patient with postdiphtheritic tabes and one patient with multiple sclerosis.

Increased cell counts (lymphocytes) were found in the majority of these fluids (Table VI). Protein contents were elevated in 5 fluids of the 12 studied. The concentrations of chlorides and sugar were normal in all fluids studied. Despite the occurrence of increased cell counts and elevated protein contents in some cases, the enzyme findings in the fluids of this group were normal for the most part. One patient with polioencephalitis (D. M.) showed deviations from the normal. Positive, though small, tryptic activity and antitryptic power were found, high tributyrinase activity was present and phosphatase was slightly increased. Abnormal lipolytic activity was found in 2 fluids. The values found were 9.0 and 5.4 units, respectively. Four other fluids had lipolytic activities somewhat higher than the normal.

#### DISCUSSION

The present work has shown that the normal cerebrospinal fluid contains beta glycerophosphatase and in many cases amylase, but that other enzymes such as trypsin, lipase, tributyrinase, and esterase are either entirely absent, or are present in small quantities in relatively few instances. The latter results are in agreement with those of Heyde<sup>1</sup> and Hiller<sup>2</sup> who studied

TABLE VI  
MISCELLANEOUS

CASE	DIAGNOSIS	CELL COUNT	TRYPSIN <i>mM.</i>	ANTI- TRYPSIN UNITS	PHOS- PHATASE <i>Mg.</i>	LIPASE <i>mM. Fatty Acid</i>	TRIPU- TYRINASE	ESTER- ASE	AMYLASE <i>mM. Maltose</i>	TOTAL PROTEIN	SUGAR	CHLO- RIDES	NON- PROTEIN NITROGEN
C. W.	Cerebral arterio- sclerosis	4	<i>Tyrosine</i> 0	0	<i>Phosphorus</i> 0.8	1.7	0	0	2.5	15	63	694	15
H. K.	Hemihypertrophy of the body	3	0	0	0.6	1.2	0	0	1.0	11	56	713	
C. W.	Huntington's chorea	10	0	0	1.7	0	0	0	2.0	31	63	696	
H. W.	Chorea	4	0	0	0.7	0	0	0	0.8				
A. M. B.	Chorea	6	0	0	0.7	0	0	0	0				
S. S.	Carbon monoxide poisoning	1	0	0		3.4		0	0	Pandy +	73	728	15
L. M.	Postencephalitic syndrome	12	0	0	0.9	0	0	0	0	56	57	734	13
T. W.	Postdiphtheritic tabes	8	0			0		0	0	56		741	23
E. Z.	Multiple sclerosis	15	0	0		9.0		0	0				
B. M.	Acute benign lym- phocytic meningi- tis	350	0	0		0		0	1.8	Pandy +	52 87	734	
E. I.	Acute benign lym- phocytic meningi- tis	280	0	0	1.1								
P. A. H.	Preparalytic polio- myelitis	93	0	0		0	0	0		42			
L. T.	Preparalytic polio- myelitis	120	0	14	0.6	1.1			0		49		
D. M.	Polioencephalitis?	9	0.03	10	2.2	0.9	2.9	0	1.8	15.5			
K. B.	Mumps meningoen- cephalitis	170	0	12		0.4		0	3.0	44			15
L. W.	Postpneumonic encephalitis		0	0	2.0	0	0	0	1.6				
A. W.*	Miliary tuberculosis	200	0	7	0.4	0	0			11			
C. T.	Typhoid	80	0		0.2	1.1	0.4	0.4		6			

\*On autopsy no meningitis was found.

proteolytic and lipolytic enzymes in normal cerebrospinal fluid and in fluids from patients with schizophrenia senile dementia and epilepsy. In tuberculous meningitis, purulent meningitis hydrocephalus brain tumor, brain abscess, and brain cyst, the cerebrospinal fluid is characterized by increase in the enzymatic activity. The question arises as to the source of the cerebrospinal fluid enzymes, both in normal and pathologic conditions of the central nervous system. Some of the possibilities will be briefly discussed.

Since enzymes are generally considered to be proteins the problem of explaining the presence in normal cerebrospinal fluid of phosphatase and amylase is closely related to the still unsolved problem of the source of the other proteins of the cerebrospinal fluid. Some authors<sup>2</sup> hold that under normal conditions proteins do not pass from the blood into the cerebrospinal fluid but originate within the cerebrospinal cavity. There is also some evidence to show that the cerebrospinal fluid acts not only as a mechanical protector of the cerebrospinal system, but also as a medium for the interchange of metabolic material between the nervous tissue and the blood system. This interchange includes the removal of waste products from the nervous tissue. Investigation of the enzymes of normal brain tissue has indicated that this tissue contains appreciable amounts of phosphatase<sup>4</sup> and small amounts of amylase,<sup>20</sup> but very little, if any, proteolytic or lipolytic enzymes.<sup>4, 20</sup> Oxidases which have been found in brain tissue in considerable quantities<sup>3, 21</sup> have also been reported in normal cerebrospinal fluid. There is therefore some parallelism between the enzymes of normal brain tissue and those of the normal cerebrospinal fluid. This may lend support to the hypothesis that the cerebrospinal fluid takes an active part in the metabolism of the central nervous system. The proteins and enzymes of the normal cerebrospinal fluid according to this theory may be liberated into the fluid during the normal metabolism of brain tissue.

On the other hand, it may be said that since the blood contains phosphatase and amylase, the enzymes of the normal cerebrospinal fluid may be derived from the blood. If this were the case one would naturally expect to find trypsin, lipase, esterase and antitrypsin in the cerebrospinal fluid, since these substances are also present in the blood. In order to account for the very small concentrations in which these substances are found in the cerebrospinal fluid, or for their absence it would be necessary to assume that the degree of permeability of the meninges and plexus differs normally for the various enzymes. There is at present insufficient experimental basis for a definite decision on this point. In connection with this problem it is interesting to note that cholinesterase has been reported present both in brain tissue and in blood, but not in the cerebrospinal fluid.<sup>22</sup> It may be that the cerebrospinal fluid contains cholinesterase inhibiting substances analogous to the antitrypsin of the blood serum, or that the nervous tissue fluid and blood fluid barriers are such that they do not permit the passage of cholinesterase into the cerebrospinal fluid. M. B. Bender has recently reported (*Am J Physiol* **126**: 180, 1939) that using the facial nerve of the cat as an indicator, he found cholinesterase activity in the cerebrospinal fluid equivalent to 1 to 2 per cent of that of blood serum.

The source of the enzymes of the cerebrospinal fluid in meningitis, both tuberculous and purulent, is also unsettled as yet. The possible sources are (a) increased permeability of the meninges, which may allow passage from the blood; (b) the liberation of enzymes by the destruction of brain tissue; and (c) the increase in the number of leucocytes in the fluid. It is difficult to determine quantitatively the relative importance of these sources. The known increased meningeal permeability in meningitis,<sup>8</sup> coupled with the fact that phosphatase, tributyrinase, lipase, and antitrypsin are found in greatly increased amount in the cerebrospinal fluid of meningitis, tends to show that these substances come at least in part from the blood. The occurrence of some breakdown of brain tissue in meningitis<sup>23</sup> would also help to explain the increased phosphatase found in meningitis. It is questionable whether this breakdown would give rise to any noticeable amounts of proteolytic or lipolytic enzymes because of the scarcity of such enzymes in brain tissue. The presence of a tryptic enzyme in the cerebrospinal fluid of meningitis is explainable, therefore, neither on the grounds of increased meningeal permeability nor in terms of destruction of brain tissue. In the purulent meningitides, where the polymorphonuclear leucocytes often amount to 98 per cent of all the cells of the fluid, the average trypsin and phosphatase activities were found to be about nine times as great as in tuberculous meningitis where the cell counts were lower and where the polymorphonuclear cells constituted only about 10 to 20 per cent of the cells. Since it has been shown<sup>24, 25</sup> that the polymorphonuclear cells are much richer in trypsin than the lymphocytes, the presence of trypsin in the cerebrospinal fluid of meningitis can probably be attributed to the polymorphonuclear cells. These cells have also been shown to have considerable phosphatase activity,<sup>26</sup> so that the high phosphatase activity of the fluid in purulent meningitis can be attributed in part to the same cells. Finally, since the antitrypsin, lipase, and tributyrinase values were quite similar in tuberculous and in purulent meningitis, but the trypsin and phosphatase values were much higher in purulent meningitis, we believe that the polymorphonuclear cells are chiefly responsible for the trypsin and phosphatase, while increased meningeal permeability is chiefly responsible for the antitrypsin, lipase, and tributyrinase.

Our work has shown that the enzymes in the cerebrospinal fluid of hydrocephalus, brain tumor, brain abscess, and brain cyst differ in general from those found in normal cerebrospinal fluid and in the fluids of meningitis. Thus it was also necessary to divide the specimens of group IV and group V into subgroups, according to whether or not the meninges or ventricles were invaded, either by a neoplasm or by hemorrhage. However, this division provides a clue to the source of the enzymes. It has been found<sup>8</sup> that brain tumors which invade the meninges or ventricles cause an increase in meningeal permeability, while tumors which do not affect the meninges or ventricles have no influence on meningeal permeability. The fluids of groups IV and V which had high enzymatic activity were obtained from patients with the following pathologic conditions: hydrocephalus due to occlusion of the foramen of Monro by a hemorrhage into the choroid plexus, hydrocephalus caused by a cerebellar medulloblastoma invading the fourth ventricle, pyo-

cephalus, cerebral subarachnoid cyst and a cerebral abscess which broke into the subarachnoid space. In all of these cases meninges or ventricles were invaded, so that there was probably increased meningeal permeability. The fact that the phosphatase, lipase, tributyltinase and antitrypsin values were high in these fluids indicates that their high enzymatic activity was due in considerable measure to the increase in meningeal permeability. In one case (cerebral abscess which broke into the subarachnoid space) the fluid was very turbid and had a high polymorphonuclear count. The trypsin and phosphatase activities of this fluid were particularly high in agreement with the idea that these enzymes, when present in appreciable quantities are derived from the polymorphonuclear cells. In 3 patients of group V—a glioblastoma of the middle fossa, an intracerebellar glioma and a cerebral abscess of undetermined location, the enzymatic activity was less than that of the patients in whom the meninges or ventricles were known to be involved. The same was true in 4 patients with relatively mild hydrocephalus. Three of these patients are alive 6 to 8 months after the studies were made; the fourth was a recent study. In some of these fluids however an interesting question is raised by the presence of high lipase and tributyltinase activities (Table IV: B, S, B, G, B, J, M, M, R, B, B, L, Table V: B, P, L, C). In these cases it is unlikely that the meningeal permeability is sufficiently increased to account for the presence of these enzymes. The leucocyte contents of these fluids are also unable to account for the high lipase and tributyltinase activities found. It is, therefore, possible that the presence of these enzymes is in some way associated with destruction of brain tissue. It is known<sup>18</sup> that in nerve degeneration, myelin is converted into droplets of unsaturated fats which are further broken down and disappear. The presence in brain tissue of large amounts of myelin and other lipoids together with the occurrence of tissue degeneration in pathologic conditions of the brain such as tumor, abscess, sclerosis, and encephalitis, make it appear reasonable to assume that the presence of lipase and tributyltinase in the cerebrospinal fluid is associated with the degeneration of brain tissue. It is possible that the high lipolytic activity found in isolated cases of multiple sclerosis, cerebral arteriosclerosis, and carbon monoxide poisoning may be explained on this basis. Whether the enzymes in question are part of the cause of the degeneration or appear as one of the effects is a question which cannot be answered as yet.

Another point of interest is brought out by our data on lipase, tributyltinase, and esterase. It can be seen from Tables I to VI that the ability of the cerebrospinal fluid to hydrolyze olive oil does not parallel its ability to hydrolyze tributyltin or ethyl butyrate. In 15 pathologic fluids both lipase and tributyltinase were found; in 15 others tributyltinase was present but not lipase; in 5 fluids, lipase was present but not tributyltinase. Eleven fluids in which tributyltinase activity was not determined showed lipolytic activity. Since esterase was found in only 5 fluids, there is no parallelism between the occurrence of lipase, tributyltinase, and esterase. Of the 5 fluids in which esterase was found, all had tributyltinase activity, but only one had lipolytic activity. It appears from this that it is necessary to distinguish carefully

TABLE VII

	GROUP I NORMAL (EPILEPSY AND MENTAL DEFICIENCY)				GROUP II TUBERCULOUS MENINGITIS				GROUP III PURULENT MENINGITIS				GROUP IV HYDROCEPHALUS				GROUP V BRAIN TUMOR, BRAIN CYST, BRAIN ABSCESS			
	NO. OF FLUIDS	MIN.	MAX.	AVG.	NO. OF FLUIDS	MIN.	MAX.	AVG.	NO. OF FLUIDS	MIN.	MAX.	AVG.	NO. OF FLUIDS	MIN.	MAX.	AVG.	NO. OF FLUIDS	MIN.	MAX.	AVG.
<i>Enzymes</i> (Expressed in units per 100 c.c. of fluid)																				
Trypsin mM. tyrosine	7	0	0	0	23	0	0.3	0.10	18	0.08	1.7	0.87	*5	0	2.32	0	6	0	1.4	
Antitrypsin units	22	0	20	3	22	0	88	28	16	0	91	31	13	26	93	60	5	0	0.15	58
Phosphatase Mg. P	13	0	1.9	0.9	14	0.0	4.7	2.2	6	9.0	22	15	4	0	7	3	5	0	18	
Lipase mM. fatty acids	34	0	0.9	0	20	0	2.4	0.4	16	0	4.3	0.7	8	5.8	66.4	5.8	5	5.8	11.4	8.6
Tributyrase mM. fatty acid	20	0	0.8	0	16	0	3.2	1.0	12	0	3.4	1.3	13	0	7.7	0	5	0	5.4	1.6
Esterase mM. fatty acid	24	0	0	0	18	0	0	0	13	0	2.9	0.5	5	0	3.9	0	4	0	0.6	0.3
Amylase mM. maltose	25	0	3.3	0.9	7	0	5.5	1.9	14	0	3.8	1.1	5	0.7	1.7	0	2	0	0	
									13	0	3.6		13		3.6		2	0	7.8	
<i>Chemical Constituents</i> (Expressed in mg. per 100 c.c. of fluid)																				
Total protein	16	5	46	21	12	50	275	145	8	32	941	262	5	456	2500		5	103	660	450
Sugar	15	45	75	56	17	0	40	21	8	0	18	10	11	5	103		5	13	207	125
Chlorides	16	687	718	719	13	569	724	656	5	650	732	681	7	36	93		3	33	56	48
Nonprotein nitrogen	14	8	20	15	6	14	26	17	4	13	21	19	10	654	890		5	686	814	729
									2	15	20	18	2	15	20		2	685	890	
									4	18	21	19	4	18	21		4	14	20	16

\*In groups IV and V the upper number refers to the first subgroup, the lower number to the second subgroup.

\*In groups IV and V the upper number refers to the first subgroup, the lower number to the second subgroup.

between the ability of the cerebrospinal fluid to hydrolyze true fats, simple esters, and esters of glycerol and low molecular weight fatty acids. The failure to do so has resulted in some confusion in the literature, since the terms "lipase" and "esterase" have both been used for the tributyrin-splitting enzyme.

Another question which arises in connection with our work is the possibility of utilizing determinations of the enzymes of the cerebrospinal fluid in diagnosis of diseases of the central nervous system. Since the enzymatic properties of the cerebrospinal fluid in tuberculous and purulent meningitis differ from those of the normal fluid the enzymes may be of use in diagnosis. Also, the fluid of tuberculous meningitis may be distinguished from that of purulent meningitis by means of the trypsin and phosphatase activities. Remembering that fluids from cases of the pre-paralytic stage of poliomyelitis and from cases of encephalitis and acute benign lymphocytic meningitis were practically normal, it may be hoped that the determination of enzymes may aid in the differential diagnosis of these diseases from tuberculous meningitis. Although it is at present too early to make more definite statements on this subject, enzyme studies have been found to be of some help in our laboratories and further work is at present in progress.

The study of hydrocephalus fluid shows that the clinical diagnosis of hydrocephalus alone, without knowledge of the cause of the condition, leads to a very limited understanding of the chemical and enzymatic findings. Even the division of hydrocephalus into such classes as communicating internal, *obstructive internal*, and *external hydrocephalus* indicates little of the etiology and course of the condition. It is necessary whenever possible to find the cause of the hydrocephalus. Our results indicate that it may be possible to distinguish on the basis of the enzymatic properties of the cerebrospinal fluid between cases where the meninges or ventricles are invaded by a neoplasm or hemorrhage, and cases where the hydrocephalus is due to congenital malformation other than a neoplasm.

Similarly, enzyme determinations may give some knowledge of the location of tumors, in particular whether or not they invade the ventricles or meninges. Finally, further work may indicate a closer relationship between the presence of lipase and tributyrinase in the cerebrospinal fluid and the extent of fatty degeneration of brain tissue.

#### CONCLUSIONS

1 Comparative studies have been made of the enzymes, cells, and chemical constituents of 113 specimens of normal and pathologic cerebrospinal fluid from 77 patients. The fluids were examined for trypsin, antitrypsin, phosphatase, lipase, tributyrinase, esterase, and amylase, as well as for cellular contents, sugar, chlorides, total protein and nonprotein nitrogen.

2 Normal cerebrospinal fluid was found to contain small amounts of beta-glycerophosphatase and amylase, and occasional traces of lipase, tributyrinase, and antitrypsin. Trypsin and esterase were never found in these fluids.

3 The cerebrospinal fluid of tuberculous meningitis had increased enzymatic activity in addition to abnormal chemical findings and elevated cell



counts. Trypsin occurred in practically all fluids from patients with tuberculous meningitis. Antitrypsin and tributyrinase were each found in about 75 per cent of the fluids. Phosphatase was found in all these fluids, generally elevated above the normal. Lipase was found in an appreciable number of fluids; esterase was never found. Amylase was elevated above the normal.

4. The cerebrospinal fluid of purulent meningitis contained trypsin and phosphatase in much greater amounts than did the fluid of tuberculous meningitis. Values for antitrypsin, tributyrinase, and lipase were similar to those found in tuberculous meningitis. Esterase was found in a few fluids. Amylase was practically normal.

5. The cerebrospinal fluid of our hydrocephalus patients fell into two groups, depending on whether or not there was a lesion of the brain which invaded the meninges or ventricles. The fluids of the former group were characterized by xanthochromia, high protein content, lipolytic activity, high antitryptic power, elevated phosphatase and tributyrinase activities. The fluids of the latter group were normal, except for the marked lipolytic activity found in some cases.

6. The cerebrospinal fluid of our patients with brain tumor, abscess, and brain cyst, also fell into two groups, depending on whether or not the lesion invaded the meninges or ventricles. The fluids of the former group were found to have generally increased enzymatic activity and antitryptic power. The fluids of the latter group showed less deviation from the normal enzyme distribution, with occasional trypsin, antitrypsin, lipase, and tributyrinase.

7. In a group of patients with miscellaneous diagnoses, the fluids were normal, except for a patient with suspected poliomyelitis and isolated cases of multiple sclerosis, carbon monoxide poisoning, and cerebral arteriosclerosis. In the last three cases, high lipolytic activity was found.

8. The possible sources of the enzyme found in normal and pathologic cerebrospinal fluids are discussed. It is suggested that the enzymes of the normal cerebrospinal fluid may result from the metabolism of brain tissue or may be derived from the enzymes of the blood. Increased enzymatic activity of pathologic cerebrospinal fluid may be due to (a) increased meningeal permeability, (b) increased number of leucocytes, or (c) destruction of brain tissue.

9. The increased lipase and tributyrinase present in the cerebrospinal fluid in pathologic conditions not resulting in increased meningeal permeability may be associated with degeneration of brain tissue.

10. The possible application of enzyme studies in the diagnosis of tuberculous meningitis, hydrocephalus, brain tumor, brain abscess, and brain cyst is considered.

#### REFERENCES

1. Levinson, A.: *Cerebrospinal Fluid in Health and in Disease*, St. Louis, 1929, C. V. Mosby Company.
2. Kafka, V.: *Die Cerebrospinalflüssigkeit*, Leipzig and Vienna, 1930, F. Deuticke.
3. Hüller, F.: *Die Beziehungen der degenerativen Veränderungen des Zentralnervensystems zu seinem Gehalt an Fett und Esterspaltenden Enzymen; sowie der Nachweis sowohl dieser Enzyme als auch von Lipidstoffwechselschlacken des Nervengewebes im Liquor Cerebrospinalis*, *Ztschr. f. d. ges. Neurol. u. Psychiat.* 109: 263, 1927.

- 4 Marchionni, A, and Ottenstein, B Diastaseuntersuchungen bei Neurosyphilis Deutsche Ztschr f Nervenl 128 83, 1932
- 5 Dochez, R A Proteolytic Enzymes and Antienzymes of Normal and Pathological Cerebrospinal Fluids, J Exper Med 11 718, 1909
- 6 Szabo, J Über Fermentwirkungen des Liquor Cerebrospinalis bei verschiedenen Geisteskrankheiten, Ztschr f d ges Neurol u Psychiat 17 145 1913
- 7 Flexner, L B The Chemistry and Nature of the Cerebrospinal Fluid, Physiol Rev 14 161, 1934
- 8 Walter, F K Die Blut Liquor Schranke, Leipzig 1929 Georg Thieme
- 9 Katzenellenbogen, S The Cerebrospinal Fluid and Its Relation to the Blood, Baltimore, 1935, Johns Hopkins Press
- 10 Anson, M L, and Mirsky, A E The Estimation of Trypsin With Hemoglobin, J Gen Physiol 17 151, 1933
- 11 Hecht, M, and Cavin, H Studies on Enzyme Action I The Estimation of Pepsin and Trypsin in Yeast, J Biol Chem 116 477 1934
- 12 Bodansky, A Phosphatase Studies II Determination of Serum Phosphatase Factors Influencing the Accuracy of the Determination J Biol Chem 101 93, 1933
- 13 Crandall, L A, and Cherry, I S The Specificity of Pancreatic Lipase Its Appearance in the Blood After Pancreatic Injury, Am J Physiol 100 266 1932
- 14 Ross J R, and Shaw, M M The Effect of Dehydration on the Pancreatic and Intestinal Enzymes, J Biol Chem 104 131, 1934
- 15 Somogyi, M Notes on Sugar Determination J Biol Chem 70 599, 1926
- 16 Somogyi, M A Method for the Preparation of Blood Filtrates for the Determination of Sugar, J Biol Chem 86 655, 1930
- 17 Van Slyke, D D The Determination of Chlorides in Blood and Tissues, J Biol Chem 58 523, 1923
- 18 Ayer, J B, Dailey, M E, and Fremont Smith, F Davis Aver Method for the Quantitative Estimation of Protein in the Cerebrospinal Fluid Arch Neurol & Psychiat 26 1038, 1931
- 19 Koch, F C, and McMeekin, T L A New Direct Versilverization Micro Kjeldahl Method and a Modification of the Nessler Folin Reagent for Ammonia, J Am Chem Soc 46 2066, 1924
- 20 Merritt, H H, and Fremont Smith, F The Cerebrospinal Fluid, Philadelphia and London, 1937, W B Saunders Company
- 21 Levinson, A, and Cohn, D Comparative Dextrose Content of Lumbar and Cisternal Cerebrospinal Fluid, Am J Dis Child 51 17 1936
- 22 Cohn, D J, Levinson, A, and McCarthy, F Physiological Variations in the Glucose Rate of Blood and Cerebrospinal Fluid Am J Physiol 103 613, 1933
- 23 Heyde, W Zur Kenntnis der Proteasen der Cerebrospinalflüssigkeit, Ztschr f d ges Neurol u Psychiat 138 536 1932
- 24 Giri, K V, and Datta, N C Brain Phosphatase Biochem J 30 1089, 1936
- 25 Takasaka, T Über den Fermentgehalt des Menschlichen Gehirns, Biochem Ztschr 184 390, 1927
- 26 Wohlgenuth, J, and Seeser, S Zur Kenntnis von der Entstehung und Zusammensetzung der Cerebrospinalflüssigkeit, Ztschr f d ges Neurol u Psychiat 13 455, 1912
- 27 Edelbacher, S, Goldschmidt, E, and Schlappe, V Über die Enzyme des Gehirns, Ztschr f Physiol Chem 227 118, 1934
- 28 Blum, E, Jakovitschuk, A, and Jarmoschkevitch, A Die proteolytische Ferments des Gehirns, Bull Biol Med exp U S S R 1 17, 1936, Physiol Abst 21 778, 1936
- 29 Krebs, H A Über die Proteolyse der Tumoren Biochem Ztschr 238 174, 1931
- 30 Gerard, R W Metabolism of Brain and Nerve, Ann Rev Biochem 6 419, 1937
- 31 Cohen, M B, and Gerard, R W Oxidizing Enzymes in Brain Extracts, Am J Physiol 119 34, 1937
- 32 Stedman, E, and Stedman, E The Relative Choline Esterase Activities of Serum and Corpuscles From the Blood of Certain Species, Biochem J 29 2107, 1935
- 33 Levinson, A Meningitis Masked by Encephalitis Symptoms, Arch Pediat 49 6, 1932
- 34 Opie, E L Intracellular Digestion, Physiol Rev 2 552 1922
- 35 Wilstatter, R, Bamann, E, and Rhodewald, M Zur Kenntnis der proteolytischen Wirkung farbloser Blutkörperchen, Ztschr f physiol Chem 185 267, 1929
- 36 Flessinger, N, and Boyer, F Les Phosphatases des Leucocytes, Enzymologia 1 172, 1936
- 37 Karsner, H T Human Pathology, Philadelphia, 1935, J B Lippincott Company
- 38 Weil, A A Textbook of Neuropathology, Philadelphia, 1933, Lea & Febiger

## NORMAL HEMATOLOGIC STANDARDS IN THE AGED\*

ISIDORE MILLER, M.D., STATEN ISLAND, N. Y.

HEMATOLOGIC standards have been made for the ages from infancy to thirty years; studies of the blood for ages beyond thirty years, and especially for the older age groups (past sixty years), have been very few and meager. Blood studies are important in both medical and surgical conditions. Before we can report variations from the normal blood count, we must know the normal values for the various ages and sex. We are presenting a hematologic study of 160 men over sixty years of age, who were inmates of a home for the aged.

Nascher<sup>1</sup> quoted the normal blood determinations in the aged as: red blood cells—3,000,000 to 5,500,000; white blood cells—4,000 to 8,000; hemoglobin—90 to 110 per cent. Leichtenstern<sup>2</sup> in 1878 found a large drop in hemoglobin from 55 to 60 years, and a rise in old age. Williamson<sup>3</sup> disagreed with Leichtenstern's findings; he stated that the report was based on too few cases. He studied the hemoglobin of 919 persons, of whom 152 (81 males, 71 females) were over 60 years. He found that the values were fairly level from the ages of 16 to 60 years (16.9 gm. hemoglobin per 100 c.c. of blood in men). Then the values declined to the age of 75 years (15.22 gm. hemoglobin); after 76 years there was a small rise (15.67 gm. hemoglobin). The values for the women past 60 years were less than for the men, but the differences were not as great as at the younger ages. Osgood<sup>4</sup> made hematologic determinations on various age groups, but his grouping did not include older men and women; in the male group, 14 to 30 years, the average red blood cell count was 5,400,000; hemoglobin, 15.8 gm. He felt that Williamson's values were accurate in relation to each other but were all too high. Wintrobe,<sup>5</sup> in his studies of erythrocyte counts and hemoglobin, which included mostly adults from 18 to 30 years and a small group of older people, concluded that there are no significant differences due to old age. He showed the erythrocyte counts, hemoglobin, and volume of packed red blood cells in men 30 to 60 years and in men over 60 years of age to be approximately the same as those of younger men. He stated that no significant differences occurred in normal adults as age advanced as long as health remained good. Price-Jones<sup>6</sup> found that an increased cell diameter in the aged was usually associated with some degree of emphysema.

Studies of the older age groups vary with the examiner and the country. Williamson,<sup>3</sup> in Chicago, found an average hemoglobin of 15.81 gm. in 81 men over 60 years. Rud,<sup>7</sup> in Denmark, found an average hemoglobin of 13.66 gm. and red blood cell count of 5,140,000 in 7 men over 60 years. Bing,<sup>8</sup> also in

\*From the New York City Farm Colony, Staten Island, N. Y.  
Received for publication, January 28, 1939.

Denmark, found an average red blood cell count of 6 100 000 in 22 men over 60 years. In a study of 50 men over 60 years in Brussels Millet and Balle Helaers' determined the average hemoglobin to be 13.3 gm, and the red cell count, 3,900,000. This suggested a deficient cardiac power or local changes in the skin and capillaries as possible causes for his high red blood cell counts, since hypertension was not present in his patients. Millet and Balle Helaers' counts were low. Various forms of anemia may have been present in some of their patients.

TABLE I  
AVERAGE HEMATOLOGIC RESULTS BY FIVE YEAR GROUPS IN 160 MEN

AGES	NO OF MEN	HGB	RBC	WBC	STAFF %	NEUTROPHILES %	LYMPHO CYTES %	MONO CYTES %	EOSINO PHILES %	BASO PHILES %
60-64	13	15.5	4.74	5,842	1	0	30	7	3	0.5
65-69	16	14.6	4.49	7,742	1	0	0	7	2	0.5
70-74	19	13.7	4.32	7,752	1.5	0	20	8	2.5	0.5
75-79	16	14.2	4.44	7,114	1.5	0	1	0	2	1
80-84	26	14.4	4.51	5,032	1	0	1	0	2	1
85-89	7	14	4.4	5,042	2	0	1	11	1	0
90-104	3	14.1	4.23	10,810	0	0	1	11	1	0
Averages		14.3	4.46	7,700	1	0	30	7.5	2.7	0.7

TABLE II  
HEMATOLOGIC STANDARDS IN OLD AGE AND A COMPARISON WITH OSOOND'S STANDARDS IN MALES, 14 TO 30 YEARS

	IN MALES—(0 TO 104 YEARS)		OSOOND'S STANDARDS IN MALES—14 TO 30 YEARS	
	AVERAGE VALUE	RANGE OF VALUES IN 95% OF CASES	AVERAGE VALUE	RANGE OF VALUES IN 95% OF CASES
Hemoglobin in grams	14.3	12-17.5	15.8	14-18
RBC in millions	4.46	3.5-5	5.4	4.0-6.2
WBC	7,700	4,000-11,000	7,400	4,000-11,500
Staff cells per cent	1.5	0.4	0.5	0.5
Neutrophiles per cent	57.5	40-75	54	33-78
Lymphocytes per cent	0	15-30	28	18-65
Monocytes per cent	7.8	1-15	4	0-9
Eosinophiles per cent	2.7	0.5	1.9	0.6
Basophiles per cent	0.7	0.2	0.5	0.2

Our studies included red and white blood cell determinations, hemoglobin, and a differential smear on 160 men with ages ranging from 60 to 104 years. In all these patients blood was obtained from the finger pulp. Our red and white blood cell counts were done with standard pipettes and a Levy-Hausser counting chamber. Hayem's solution was used for the red blood cell and Turk's solution for the white blood cell counts. The hemoglobin determinations were made with the Hellige wedge hemometer. Our differential smears were stained with Wright's solution and 200 cells were counted on each slide. These determinations were all made in the summer months (June, July, and August) and in the late afternoon.

Table I shows the average hematologic results by five year groups. Table II gives our average hematologic values for the 60 year and over group, and

presents the range of values in over 95 per cent of these patients. We also compared our average findings with those of Osgood<sup>4</sup> which he obtained in his younger males.

#### DISCUSSION

In the older age groups, senile retrogressive changes, arteriosclerosis, pulmonary emphysema, and hypertension are present to a greater or lesser degree. The degenerative changes are an integral part of the senescent process, and when we speak of normal old men we include those with changes which are peculiar to their age and exclude those with inflammatory or neoplastic pathologic processes. In our present study we included ambulatory patients who were institutionalized for custodial care and whose physical examination was negative except for changes incidental to old age.

The average red blood cell count in our series was 4,460,000 per c.mm., with a range from 3,500,000 to 5,500,000. Both Osgood and Wintrobe in their younger subjects found an average red blood cell count of 5,400,000, with a range of values from 4,600,000 to 6,200,000. The average red blood cell count in the aged is 81 per cent of the average found in the younger ages; in other words, the count per c.mm. is one million less for the older age groups than for the normal adults. Hemoglobin diminishes proportionately to the decrease in red blood cells. We found an average hemoglobin of 14.3 gm. per 100 c.c. of blood, with a range from 12 to 17.5 gm. Osgood found an average hemoglobin of 15.8 gm., and Wintrobe, of 16 gm.; their range of values was 14 to 18 gm. There was an average decrease of 1.5 to 1.7 gm. of hemoglobin per 100 c.c. of blood in comparing the determinations in the young and the old. On the basis of a 5,000,000 red blood cell count the value for 100 per cent hemoglobin in our study was 16 gm. With Osgood's figures the equivalent of 100 per cent hemoglobin is 14.7 gm. Wintrobe suggested the use of 14.5 gm. hemoglobin as 100 per cent.

TABLE III

HEMOGLOBIN AND THE ERYTHROCYTES IN RELATION TO BLOOD PRESSURE

	NO. OF MEN	HEMOGLOBIN IN GRAMS	RED BLOOD CELLS IN MILLIONS
Hypotensive cases	6	14.3	4.37
Normal blood pressure cases	83	14.3	4.50
Hypertensive cases (140-160)	17	14.4	4.43
Hypertensive cases (160 and over)	54	14.2	4.43

An increase in red blood cells occurs in people living in high altitudes, in chronic pulmonary diseases, and in myocardial insufficiency. The altitude of the institution where our blood studies were made is 300 feet above sea level. This elevation is not sufficient to affect our results. We omitted from this study all patients with cardiac murmurs, dependent edema, dyspnea, cyanosis, pulmonary fibrosis, and pulmonary tuberculosis. We included hypertensive patients. As Table III indicates, we divided our patients into three main groups according to their blood pressure. The normal blood pressure group included those with systolic pressures between 120 and 140 and diastolic pressures of 90 or under. The hypertensive group included those

with systolic pressures over 140 and diastolic pressures over 90. In the hypotensive group were those with systolic pressures under 120. The hypertensive group was subdivided into those with systolic pressures between 140 and 160 and those with systolic pressures of 160 and over. The normal blood pressure group had the highest red blood cell count (4,500,000), and the hypotensive group, the lowest (4,370,000). The 140 to 160 blood pressure group had the highest hemoglobin average (14.4 gm.), while the 160 and over group had the lowest (14.2 gm.). The differences in the red blood cell count and in the amount of hemoglobin in the various groups were slight. This finding differs from the results obtained by Doles<sup>10</sup> who reported an increase in the red blood cell count with hypertension.

We can only surmise the causes of anemia in old age. Chronic low grade infections, not sufficient to alter the white blood cell count, may be a factor. In a study of blood sedimentation rates in the aged,<sup>11</sup> we have shown that one third have a sedimentation rate above normal. Oral sepsis is prevalent, pyorrhea and carious teeth being the main causes. Prostatic hypertrophy is common in this age group. This produces an obstruction to the urinary outflow and stagnation of urine in the urinary bladder with an increased possibility of infection and urinary decomposition. Bronchiectasis may also play a part in this picture. Taylor and Schwartz<sup>12</sup> in a roentgenologic study of 125 apparently normal men from this institution found interstitial changes suggesting bronchiectasis in 36 cases (27 per cent). The stagnant bronchial secretion may be a fertile soil for chronic sepsis. Nephrosclerosis, the common kidney finding in old age, may also play a part in the production of the anemia. Decrease in the cellularity of the red bone marrow with advancing age may be another factor.

The average leucocyte count was 7,700 cells with a range from 4,000 to 13,000. Osgood, in his younger group, found an average of 7,400 cells, ranging from 4,500 to 11,500. The minimum and average counts agreed fairly well, but the old age group showed a higher maximum value.

The differential smear gave average percentages within normal limits. A comparison of our findings with those of Osgood is presented in Table II, the average percentages and the range of values for the various white blood cells are shown. Thirty-three of our patients had an eosinophile count of 5 per cent and over. The highest was 16 per cent. We believe that this was due to the skin changes that occur in old age. Atrophy, seborrhea, dryness and pruritus are common dermatologic findings in the aged.

#### CONCLUSIONS

1 The red blood cells per cubic millimeter of blood are diminished in old age. The average red blood cell count is 4,460,000. Chronic low grade infection, nephrosclerosis, and decreased cellularity of the red bone marrow in old age may be causative factors. Hypertension did not produce an increase in the red blood cells.

2 The hemoglobin per 100 cc. of blood is decreased in the aged, the average being 14.3 gm. The decrease in hemoglobin is proportional to the decrease in red blood cells.

3. The white blood cells are within normal limits.
4. Differential counts are within normal limits.

The author wishes to express his appreciation to Miss Martha A. Klose for her technical assistance.

#### REFERENCES

1. Nascher, I. L.: *Geriatrics, The Diseases of Old Age and Their Treatment*, Philadelphia, 1914, P. Blakiston's Son & Co.
2. Leichtenstern: Quoted by Williamson, C. S.: *Influence of Age and Sex on Hemoglobin*, *Arch. Int. Med.* 18: 505, 1916.
3. Williamson, C. S.: *Influence of Age and Sex on Hemoglobin*, *Arch. Int. Med.* 18: 505, 1916.
4. Osgood, E. E.: *Normal Hematologic Standards*, *Arch. Int. Med.* 56: 849, 1935.
5. Wintrobe, M. M.: *The Erythrocyte in Man*, *Medicine* 9: 195, 1930; *Blood of Normal Men and Women*, *Bull. Johns Hopkins Hosp.* 53: 118, 1933.
6. Price-Jones, C.: *The Signs of Red Blood Cells in Emphysema*, *J. Path. & Bact.* 24: 326, 1921.
7. Rud, E. J.: *Red Corpuscles and Their Variations*, *Acta med. Scandinav.* 57: 142, 1922; 57: 325, 1922.
8. Bing, H. I.: *Number of Red Blood Corpuscles at Different Ages and Under Different Conditions*, *Acta med. Scandinav.* 53: 833, 1921.
9. Millet and Balle-Helaers, E.: *Hemoglobin Values in the Aged*, *Sang.* 6: 735, 1932.
10. Doles, H. M.: *The Erythrocyte and Its Relation to Blood Pressure*, *Virginia M. Monthly* 62: 489, 1935.
11. Miller, I.: *Blood Sedimentation Rates in Middle-Aged and Old People*, *J. LAB. & CLIN. MED.* 21: 1227, 1936.
12. Taylor, H. K., and Schwartz, S.: *Roentgen Findings of the Thorax in the Aged*, *Quart. Bull., Sea View Hosp.* 3: 250, 1938.

# LABORATORY METHODS

## A SHORT CUT IN GAS ANALYSIS\*

P. S. COTTON, D.Sc. SYDNEY AUSTRALIA

IN CURRENT methods of gas analysis where more than one gas must be estimated, only the first gas dealt with can be absorbed in one unbroken series of visits to its appropriate absorption pipette. In the case of each gas subsequently estimated, the dead space † in each of the absorption pipettes previously visited must be flushed out several times (usually three separate times) for the complete elimination of that gas.

Suppose, for example, that certain gases *a*, *b*, and *c* in *II* of Fig. 1, are to be removed in order from a gas sample measured in burette *Bt* by visits to absorption pipettes *A*, *B*, and *C*, respectively. The procedure would run as follows:

1 The gas sample, after initial measurement of volume is driven over into *A* the requisite number of times, say  $N_1$ , and the reduced volume read.

2 Next the gas is driven into *B* a certain number of times, say  $N_2$ , then returned to *A* to flush out the dead space, driven back to *B* for another series of visits,  $N_3$ , returned to *A* twice more with subsequent series of visits,  $N_4$  and  $N_5$ , to *B*. The reduced volume is then read on returning to the graduated burette.

3 The gas must now be driven over into *C* with correspondingly more complicated interruptions for the purpose of revisiting *A* and *B* to remove the last traces of the gas *c*. This involves a series of interrupted visits  $N_1, N_2, N_3, N_4$  to *C*, with flushings of the dead spaces in *A* and *B* in between each constituent series of these visits. Thus to complete the analysis some nine series of trips to absorbing fluids must be made, together with several flushings of capillary spaces.

Quite apart from the loss of time due to interrupting the visits to an absorption pipette, the total number of trips involved is much greater than that necessary to completely absorb that constituent from one undivided sample of gas. Thus if 15 trips to the pyro solution were necessary to remove quantitatively the oxygen from a gas sample driven without interruption into and out of *C*, then not less than 35 to 40 total visits would be required for this purpose in the routine analysis to ensure complete absorption of the oxygen from the flushings of the dead spaces in *A* and *B*.

If the burette in question has a capacity of about 10 mls it does not in general require a great many trips to pyro to remove adequately the oxygen,

\*From the Fatigue Laboratory, Morgan Hall, Harvard University, and the Department of Physiology, University of Sydney.

Received for publication September 30 1938

†By the dead space is meant the space in each absorption pipette *A*, *B*, *C* above the level of the fluid meniscus in. In the pipettes *II* of Fig. 1 it also includes the volume of the oblique bore of the stopcock leading to the pipette in question.



but if, on the other hand, sufficiently accurate analyses are required to demand the use of a pipette with a capacity of 20 to 25 mls, the complete absorption of oxygen from this larger gas sample requires a more than proportionate number of trips to pyro. It was just such a tedious procedure requiring the estimation of carbon dioxide, acetylene, and oxygen from a gas sample that led to this attempt to shorten the process.

The simplest possible procedure in such an analysis would be realized if the absorption of each gas could be accomplished by one unbroken series of visits to its appropriate pipette. This could be achieved if *each constituent of the gas mixture could be absorbed from all but an accurately known small fraction of the total gas sample*. This could in turn be performed if the following conditions could be fulfilled.

- (1) That an accurately known amount of the gas sample could be separated from the rest and left behind in the dead space of any given pipette after the absorption of one gas in it was complete.
- (2) If subsequently either (a) the whole of any unabsorbed gas remaining there in the first place was finally left within that pipette until the next analysis or (b) a completely negligible amount of any unabsorbed gas remaining there in the first place was finally left within that pipette until the next analysis.

By an appropriate redesigning of the standard Haldane and Haldane-Grollman apparatus, these conditions were completely and quantitatively fulfilled. In the case of the acetylene fraction of the gas sample conditions 1 and 2 (b) were fulfilled, and in the case of the oxygen fraction conditions 1 and 2 (a). In an alternative method of using the apparatus conditions 1 and 2 (a) can be fulfilled in the case of both these gases.

*Detail of the Changed Design.*—In Fig. 1, *I* represents the general design of the standard Haldane apparatus, *A* being the pipette for the absorption of carbon dioxide and *B* the pipette for the absorption of oxygen. The type of three-way tap above *A* and *B* is shown in cross section enlarged below (*III*). In Fig. 1, *II* represents the Haldane-Grollman apparatus with pipettes *A* for carbon dioxide, *B* for acetylene, and *C* for oxygen. The type of three-way tap above *A*, *B* and *C*, is shown in *IV* in longitudinal section.

In the first type of apparatus the dead space is equal to about 1/50 of the total gas sample, and in the second type it is still more, due to the additional length of horizontal capillary which is the inevitable result of using the oblique stopcock. This dead space could be reduced in either type of apparatus by setting the menisci higher by means of the adjustable clamp usually employed with such apparatus. The reduction so effected, however, would still leave too large a dead space with the caliber of tubing currently used, and, moreover, with the oblique stopcock the smallest capillary tubing that could be employed for use with both gas and liquid would still leave that residual volume too great. (The horizontal length of capillary cannot be reduced to zero.)

Since it is highly desirable to employ the oblique type of stopcock in preference to the three-way type, as much less liable to develop leaks with extended use, this disadvantage of excessive dead space was obviated by designing a stop-

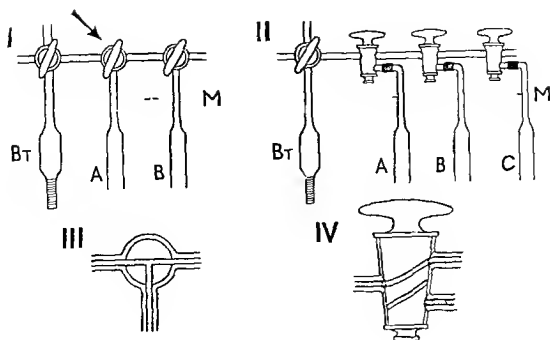


Fig. 1

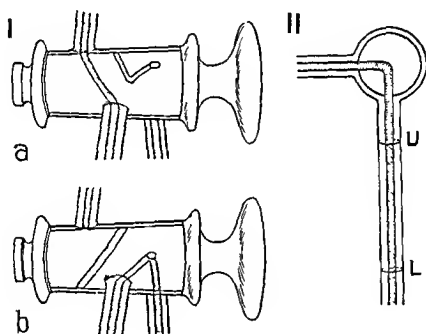


Fig. 2

cock to combine the separate advantages of the two types shown. This is illustrated in Fig 2, I, which was drawn from photographs of such a stopcock taken from the direction shown by the arrow in Fig 1. In *b* the tap is turned  $180^\circ$  from the position *a*. This stopcock may be described as a modification of the ordinary oblique tap, produced by accomplishing the imaginary act of bending one of the parallel bores at right angles at the center. This permits making a vertical lead out tube coming straight down from the stopcock and avoiding the waste horizontal length shown in Fig 1, II. Further, by reducing the bore of the capillary, the residual dead space (remaining when the visits to a pipette are completed and the liquid meniscus finally adjusted) was cut down to an extremely small volume shown by the stippled space in II of Fig 2. This, of course, includes the right angled bore of the stopcock.

By varying the capillary bore in different experimental pipettes, an optimum diameter was finally secured which was sufficiently small for the purpose

in hand and yet sufficiently large for use with fluids. In a similar way, starting with a stopcock drilled with too fine a bore, and involving difficulties both with regard to undue resistance to the flow of gas and a tendency to become partially blocked with tap grease, this bore was enlarged by drilling several times, until a diameter compatible with all desiderata was reached.

In the case of the lead out capillary (*UL* of Fig. 2) an optimum diameter was found to be about 1.5 mm., while the corresponding dimensions in the case of the stopcock were finalized at about 1.8 mm. diameter for the straight bore and about 1.7 mm. for the right angled bore.

*Detail of the Analytical Procedure* (Procedure A).—The following description is given with reference to the general layout of Fig. 1, *I*. The reader is asked to imagine that the stopcocks shown here are replaced by the type illustrated in Fig. 2, *I*, and that Fig. 2, *II*, represents the capillary joining each absorption pipette to the stopcock above it. After the absorption of the carbon dioxide in pipette *A* and measurement of the changed volume with the meniscus set at the lower level *L*, the adjusting screw is used to raise the fluid level to the upper mark *U*, just a few millimeters below the stopcock. This stopcock is then turned to the position shown in *I a* of Fig. 2, and the gas driven into and out of pipette *B* a sufficient number of times (previously determined) to completely absorb the acetylene. The menisci of *B* and *A* are then set to their corresponding *L* marks\* and the second reduced volume is read in the burette.

Next, before proceeding to drive the gas into *A* the fluid levels in *A* and *B* are both raised to the *U* levels and the stopcocks are appropriately turned. When sufficient visits to pyro have been made, the final procedure must be carried out rigidly as follows:

1. The pyro meniscus is set to its mark.
2. The stopcock is turned to communicate with *B* and the bulk of the nitrogen driven into that pipette to dilute the contents of the residual dead space and so reduce the percentage of oxygen to a negligible amount.
3. The fluid meniscus is set to the *L* level in *B* and the same procedure as 2 repeated in the case of pipette *A*.
4. The final volume is read in the burette and the uncorrected percentages of carbon dioxide, acetylene, and oxygen are calculated.
5. The acetylene percentage is then increased† by 1/500 and the oxygen percentage by 1/250 (corresponding exactly in each case to the volume of gas locked up in the residual dead spaces), and the analysis is complete.

The following example illustrates in the order of the maximum corrections involved, since the percentage amount of oxygen in the samples analyzed seldom exceeds 22 per cent. The uncorrected analysis gave 5.37 per cent carbon dioxide, 10.54 per cent acetylene, and 22.19 per cent oxygen.

\*The setting of the meniscus of the capillary attached to the control burette is assumed at each reading of the burette, but omitted from the text to simplify the description.

†These convenient ratios did not fall out by chance when the apparatus was constructed. They were altered several times as a consequence of redrilling the stopcocks to secure the optimal bore. The correction concerned was then about 1/440 for each pipette. By using fine wire rings around the capillary for graduation marks, exact positions were found to yield the more suitable ratios quoted above, and so permit of calculation by immediate inspection. The method for accurately determining the volumes of the residual dead spaces is described in the Addendum.

The correction for the acetylene amounts to  $1/500$  of  $10.54$  per cent, i.e.,  $0.021$  per cent or  $0.02$  per cent to the nearest significant figure, while the correction for the oxygen amounts to  $1/250$  of  $22.19$  per cent, i.e.,  $0.088$  per cent or  $0.09$  per cent to the nearest significant figure.

Further, since the total volume of the capillary system of the manifold is less than  $1/50$  of the burette volume the effect of the final flushing procedure is to remove all except  $1/50$  of these correction residuals. Inasmuch as this final residue has a maximum value of  $1/50$  of  $0.09$  per cent it will be obviously quite negligible in respect to the succeeding analysis.

To illustrate the accuracy of the method a series of five consecutive pairs of duplicate analyses, occurring in routine estimation of the cardiac output by Grollman's acetylene method, are given in Table I. The duplicate analyses were made by two separate machines.

TABLE I\*

	MACHINE A	MACHINE B
Carbon dioxide	6.48	6.48
Acetylene	8.62	8.63
Oxygen	15.5	15.37
Total	30.60	30.48
Carbon dioxide	5.67	5.65
Acetylene	9.59	9.60
Oxygen	16.63	16.66
Total	31.91	31.91
Carbon dioxide	1.94	4.93
Acetylene	10.38	10.39
Oxygen	17.46	17.47
Total	32.78	32.78
Carbon dioxide	6.75	6.76
Acetylene	8.25	8.25
Oxygen	14.64	14.64
Total	29.64	29.64
Carbon dioxide	5.57	5.57
Acetylene	7.06	7.05
Oxygen	16.42	16.44
Total	29.05	29.06

\*It should be noted by those interested in analyses of acetylene that the degree of accuracy illustrated in Table I is not obtainable unless the mercury in the burette has been thoroughly cleaned. When the mercury surface becomes dirty the analytical accuracy falls off remarkably. This has been observed by others but no complete explanation has yet been given. The series in the table has therefore been selected from this point of view but it is a fair representation of the accuracy of which the method is capable.

*Further Simplification of the Analysis (Procedure B)*—When a series of analyses are being made a simplification of the foregoing procedure may be adopted with advantage. In this the final flushing of the absorbing pipettes with nitrogen may be omitted without loss of accuracy provided the following routine be strictly observed:

1. After the pyro meniscus has been set the stopcock is turned to pipette B, when the elevated liquid column immediately drops. This is quickly pushed down to the lower engraved mark by means of the adjusting screw, the meniscus

is set, and the stopcock turned off to an oblique position. The effect of this procedure is to carry the oxygen within the residual dead space down to the lower portion of the gross dead space and to prevent effectually its diffusion back into the capillary of the manifold. The same procedure is then repeated in the case of pipette A. In this way these two small oxygen fractions are kept within the system, and become absorbed during the next analysis. Since the error thereby introduced is only  $1/250$  of the difference in the percentages of oxygen in the two samples, it is clear that no correction need be applied unless the difference in composition amounts to about 3 per cent. This seldom happens in a series of analyses. If under exceptional conditions a greater difference in two samples does occur, it is merely a matter of a moment's inspection to make an accurate correction. Thus if an analysis yielding 20 per cent of oxygen followed another showing 14 per cent of oxygen, the former would require a correction of  $1/250 (20-14)$ , i.e., 0.02 per cent. In order to test the accuracy of the procedure just described (Procedure B), an experimental mixture of carbon dioxide and oxygen was prepared, well mixed, and several samples were taken off. These were analyzed in duplicate in each of three machines by the short-cut method just described and in one machine by the standard Haldane procedure. The results are shown in Table II.

TABLE II

TYPE OF ANALYTICAL PROCEDURE		ANALYSIS NO.		MEAN	MACHINE
		1	2		
Standard	Carbon dioxide	3.67	3.65	3.66	A
Haldane	Oxygen	16.83+	16.84	16.84	
Procedure	Carbon dioxide + Oxygen	20.50+	20.49	20.50	
Short cut	Carbon dioxide	3.67	3.68	3.67+	A
Procedure B	Oxygen	16.83	16.84	16.83+	
	Carbon dioxide + Oxygen	20.50	20.52	20.51	
Short cut	Carbon dioxide	3.66+	3.66	3.66	B
Procedure B	Oxygen	16.83+	16.84	16.84	
	Carbon dioxide + Oxygen	20.49+	20.50	20.50	
Short cut	Carbon dioxide	3.67	3.66	3.66+	C
Procedure B	Oxygen	16.84	16.86	16.85	
	Carbon dioxide + Oxygen	20.51	20.52	20.51+	

## SUMMARY

1. A method is described to obviate the flushing of the dead spaces in routine gas analysis.

2. In the case of a triple gas analysis, such as for carbon dioxide, acetylene, and oxygen, the number of visits to absorbing solutions is reduced to the order of half the usual number.

3. The error introduced thereby is only a small fraction of the average error of the gas analysis itself.

4. The method depends upon special points of construction in the gas analysis apparatus which are described in full.

*Addendum* The determination of the volume of the residual dead space was estimated in the following way

1 Pure oxygen was taken into the machine and used to flush out both pipettes *A* and *B* after which the menisci were raised to the *U* marks and the stopcocks turned to lock up practically pure oxygen in the residual dead spaces

2 The oxygen in the burette was then replaced by air and by sufficient trips to pyro all of that gas removed so as to leave nitrogen only

3 By appropriate turning of the stopcocks all 3 menisci were then set to the *L* marks and the burette volume was read

4 By adequate refushing of the system and trips to pyro all the oxygen now in the system was absorbed and the diminished burette volume read

5 The difference of the two volumes gives the sum of the residual dead spaces Since the dimensions of these are almost identical each individual dead space is one half the total so obtained

I wish to express my thanks to Arthur H. Thomas Co. of Philadelphia for their cooperation in making all the stopcocks and most of the experimental glassware required for the work. They now make the complete apparatus

---

## DETECTION OF BLOOD BY MEANS OF CHEMILUMINESCENCE\*

FREDERICK PROESCHER, M.D. SAN JOSE, CALIF., AND  
A. M. MOODY, M.D., SAN FRANCISCO, CALIF.

---

THE chemiluminescence of certain organic compounds has aroused considerable academic interest in recent years. The two most active substances are 3 aminophthalhydrazide (luminol) and dimethyldiacrydil nitrate which, by oxidation, exhibit an intense luminescence that so far surpasses all such known phenomena.

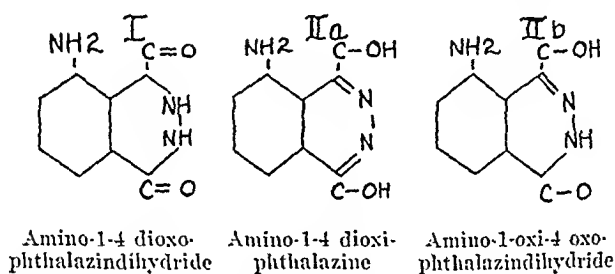
Glen and Pfannstiel,<sup>1</sup> and Wegler<sup>2</sup> have recently made a thorough investigation of the 3 aminophthalhydrazide and other substituted hydrazides, and described new methods for their preparation. The 3 aminophthalhydrazide was first synthesized by Schuntz,<sup>3</sup> under the direction of Curtius, from the ester of the hemimellitic acid. Later on Curtius and Semper<sup>4</sup> obtained it from 3 nitrophthalacid 1 ester by boiling with hydrazine hydrate. They noticed the very strong bluish fluorescence in acid solution, but the most interesting property, the chemiluminescence, escaped their observation.

The 3 aminophthalhydrazide exhibits an intense blue chemiluminescence, by oxidation in alkaline solution, which was observed for the first time by Lommel in the Research Laboratories of the I. G. Farbenwerke in Leverkusen, Germany. Lommel did not publish his observation but interested Albrecht<sup>5</sup> in studying the phenomena from a physicochemical standpoint. Harvey<sup>6</sup> found

\*From the Santa Clara County Hospital, San Jose and the St. Francis Hospital, San Francisco.

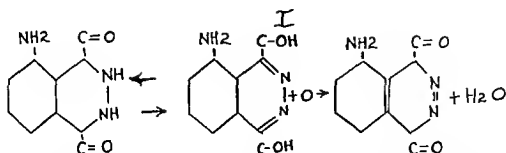
that chemiluminescence of the alkaline solution of 3-aminophthalhydrazide appears by anodic oxidation, or through activated molecular oxygen (activated by metals). Harvey considered the appearance of luminescence a criterion of active oxygen.

In 1935 Hundress, Stanley, and Parker<sup>7</sup> published a method for the preparation of 3-aminophthalhydrazide, and named the substance luminol. Harris and Parker<sup>8</sup> made physicochemical measurements of the quantum output of the chemiluminescence produced by luminol. According to Glen and Pfannstiel, a convenient method for the preparation of pure 3-aminophthalhydrazide consists of boiling benzisoxazolone-4-carboxylic acid with a strong aqueous solution of hydrazine. After diluting with water and cooling, yellow crystals of pure hydrazide are obtained. If the benzisoxazolone-4-carboxylic acid is boiled for a longer time with hydrazide, or the yellow hydrazide treated with boiling hydrochloric acid, pure white hydrazide is obtained in good yields. The light yellow 3-aminophthalhydrazide is a phthalazine derivative existing in three tautomer forms.

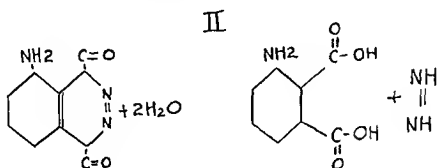


In dilute acids it is only slightly soluble. Such solutions probably contain form I and give a strong blue fluorescence. The addition of oxidizing agents like hypochlorites, does not produce luminescence. Forms IIa and IIb exist in alkaline solution. The 3-aminophthalhydrazide is easily soluble in alkali and forms well-defined monoalkali salts. The solution exhibits a very weak green fluorescence but gives an intense blue luminescence by oxidation with sodium hypochlorite. The aqueous solution of 3-aminophthalhydrazide gives a strong blue fluorescence, as do also the solid substances; forms I and II are probably combined into a salt. Chemiluminescence is only produced by oxidation in alkaline solution of form II. Originally calcium hypochlorite was used as an oxidizing substance, but other oxidizing substances like potassium ferrieyanide, ammoniacal copper solution, manganese peroxide, colloidal platinum, blood and vegetable peroxidases produce the same effect. Peroxide oxidizes slowly and produces a weak but continuous luminescence which increases with the elevation of temperature. Luminescence may continue for months at room temperature, but is weak in intensity. Persulfates have a similar action.

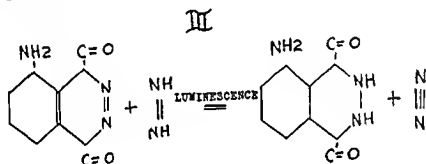
The 3-aminophthalhydrazide is rapidly oxidized with the formation of aliphatic acids and becomes brown in color. If the amino group is acetylated, acetyl 3-aminophthalhydrazide is formed and does not give a brown discoloration by oxidation; the luminescence is slightly green but very intense. The first phase of the reaction is the oxidation of the hydrazide to an azo compound.



The azo compound has not been isolated so far. During its formation energy is stored. The azodiazoles are not water stable and hydrolyze almost completely in the presence of alkali.



The hypothetical intermediary product then reacts with another molecule of the azo compound:



The last two equations demonstrate the luminescent process. The oxidation, therefore, is not the direct cause of the luminescence but only serves to produce the azo compound. If this compound is formed, luminescence only appears after the addition of alkali. The oxidation of 3-aminophthalhydrazide forms an azo compound which is again transformed into an activated fluorescent aminophthalhydrazide.

Albrecht had already noted that blood as well as fresh potato juice, produced a strong luminescence in the presence of hydrogen peroxide. Glen and Pfannstiel confirmed Albrecht's observation. Boiled vegetable peroxidases did not give a luminescence. Pure hematin produced the most brilliant luminescence.

Upon the suggestion of Glen and Pfannstiel Specht<sup>9</sup> called attention to the forensic detection of blood stains by means of chemiluminescence. Specht's extensive investigations with fresh and old blood stains, as well as other stains which simulate blood, like colored preserves, milk, coffee stains, organic and inorganic dyes (eosin, scarlet red, etc.), sperm, saliva, urine, feces, and other blood fluids, did not give a luminescence. Wallpaper, fabrics, leather, moldy bread, fungous cultures, oils and oil colors, varnish, mineral



oils, wax, shoe cream, soil, clay, sand, various stones, wood, grass, leaves, metals (copper, iron, brass, lead and zinc), did not produce a luminescence. Other metal oxides, especially rust, which in practice is often contaminated with blood and reacts strongly with hydrogen peroxide, do not react with luminol. Specht demonstrated the practicability of the method by spraying bushes, stone walls, rusty fences, etc., with blood; after exposure to sunlight and rain, luminol made the blood stains distinctly visible so that they could be photographed in the dark. Blood could also be detected in water, soap water, and sewage. A weak luminescence may be produced in the presence of hypochlorites, but with the careful addition of sodium hydroxide a momentary marked lighting will appear. Blood stains on hands could also be detected easily.



Fig. 1.—Model experiment with hematin. Photographed with orthochromatic film, sensitized with ammonia. Exposure time: forty seconds.

The 3-aminophthalhydrazide which we used in our experiments was obtained from the Eastman Kodak Co. It is a yellow, amorphous substance, easily soluble in a sodium carbonate solution. It is probably a mixture of yellow and white hydrazide and exhibits a greenish-yellow fluorescence in ultraviolet light. This substance is difficult to dissolve in boiling hydrochloric acid, and the acid solution does not fluoresce. The alkaline solution exhibits a brilliant luminescence with sodium hypochlorite, potassium ferricyanide, ammoniacal copper solution, manganese peroxide, hematin, and vegetable peroxidases.

Specht used a 0.1 per cent solution of luminol in 5 per cent sodium carbonate with the addition of 15 per cent superoxydol (Merck) or 0.1 per cent luminol in 100 c.c. of a 0.5 per cent solution of sodium peroxide, in his experiments. The concentration of the sodium carbonate can be reduced to 0.5 per cent without impairing the brilliance of the luminescence. The expensive superoxydol can be replaced by the cheaper 3 per cent commercial hydrogen peroxide by adding 10 or 15 per cent to an alkaline luminol solution. The brilliance of the luminescence diminishes in a short time (ten to fifteen min-

utes) with a soda alkaline solution of luminol, but when luminol is dissolved in a filtered saturated solution of calcium hydroxide, the luminescence is prolonged.

We can confirm Speelt's findings as to the negative luminescence of the various above mentioned substances. While the test is primarily a presumptive one for blood, it greatly facilitates the detection of blood stains as large areas of suspected material can be examined rapidly. We detected blood stains on paper, fabrics, and iron pipes which had been exposed to sunlight and air for three years. Three-year-old putrefied blood also gave brilliant luminescence. Dried and decomposed blood gives a stronger and more lasting reaction than fresh blood. If the luminescence disappears, it may be reproduced by the application of a fresh luminol-hydrogen peroxide solution. Dried blood stains may be made luminescent many times. Fresh blood stains which have been allowed to dry for a short time, may be made more luminescent if they are first sprayed with a 1 or 2 per cent solution of hydrochloric acid, which hydrolyzes the hemoglobin and produces acid hematin. Application of the alkaline luminol solution will give a strong luminescence.

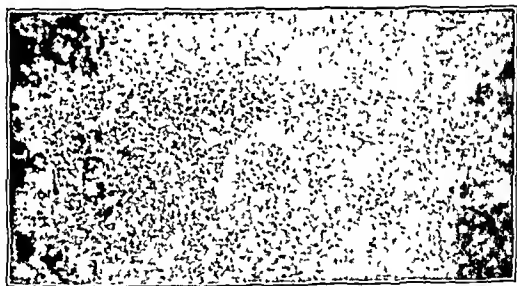


Fig 2—Rusty steel plate with oil paint and varnish spots contaminated with blood stains ten days old. Photographed by daylight.

Carbonized blood can also be detected if extracted with boiling dilute hydrochloric acid (10 per cent), the filtrate made alkaline and mixed with luminol and hydrogen peroxide. The remaining carbon, if still containing a sufficient amount of hematin, will also react with luminol if alkalinized. The chemical reaction that takes place between luminol, hematin, and hydrogen peroxide, is as follows: The nascent oxygen of the hydrogen peroxide is transferred to the bivalent iron of hematin which oxidizes the luminol and produces the luminescence.

Luminol reacts with animal, as well as with human, blood and is, therefore, only a general reagent for peroxidases. The advantage of luminol for the detection of blood, is its extreme sensitiveness; hematin can be detected in a dilution of 1:100,000,000. If blood stains are detected with luminol, they must be differentiated further as to their specific nature. Luminol does not interfere with spectroscopic, chemical, or precipitation tests, for the definite

identification of blood. Hematin and hemochromogen crystals of dried blood were obtained after repeatedly treating blood with luminol. The precipitin test can be applied if the blood is not decomposed.

*Reagents.*—Dissolve 0.1 gm. of luminol in 100 c.c. of a 5 per cent solution of chemically pure sodium carbonate. This solution may be kept indefinitely. Add 15 to 20 c.c. of a 15 per cent superoxydol solution (Merek) or the same amount of a 3 per cent commercial hydrogen peroxide to 100 c.c. of the luminol solution. If sodium peroxide is used in place of hydrogen peroxide, add 1 gm. to 100 c.c. of the luminol solution. Solutions should be made with distilled water and stored in clean bottles. The hydrogen peroxide or sodium peroxide should be added just before making the test. Traces of metal salts or free halogens will produce a slight luminescence, but this does not interfere with the test.

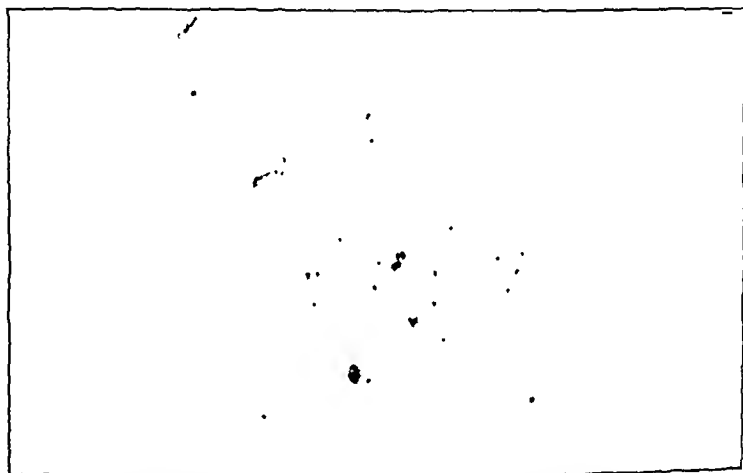


Fig. 3.—Same as Fig. 2 sprayed with luminol peroxide and photographed in darkroom. Orthochromatic film. Exposure time: two minutes.

*Procedure.*—To detect fresh blood in solution, it is preferable to transform hemoglobin into hematin. The solution is acidified with a few drops of chemically pure concentrated hydrochloric acid, boiled for a few minutes, cooled, and alkalized with a chemically pure sodium carbonate solution. Boiling will destroy vegetable peroxidases which also react to luminol. Add 5 to 10 c.c. of the luminol-peroxide reagent to the alkalized solution. If blood is present, a brilliant luminescence will immediately appear. The duration of the luminescence depends upon the amount of blood present. If the luminescence fades, it may be reproduced by adding a few cubic centimeters of hydrogen peroxide. This test is best performed in a darkroom.

To detect blood on paper, fabric, leaves, etc., it is more convenient to spray such areas with a 1 to 2 per cent solution of hydrochloric acid from a glass atomizer, to be followed in ten or fifteen minutes by spraying first with the sodium carbonate solution and then with the luminol-peroxide reagent. When blood is present, the smallest droplets become luminescent in the dark. If the luminescence fades, it may be reproduced by allowing the objects to dry and again spraying with the reagent.

To detect blood in carbonized material, it should be finely ground and extracted with a 10 to 15 per cent solution of boiling hydrochloric acid. After filtration of the undissolved residue, the extraction may be repeated several times, the combined filtrates evaporated to a small volume, and the solution alkalinized and treated as previously described. The insoluble residue may be suspended in a 5 per cent sodium carbonate solution and mixed with the reagent. If the hematin is not completely extracted, the undissolved particles will produce a distinct luminescence.

*Conclusion*—The 3 aminophthalhydrazide (luminol) in soda or earth alkali solution, in the presence of small amounts of hydrogen peroxide, is a general reagent for peroxidases like leuco-malachite green, benzidine, phenolphthalein, etc. While the leucoaniline bases give a distinct color reaction in combination with hydrogen peroxide, luminol exhibits a brilliant luminescence in the dark. The luminescence can be photographed with orthochromatic plates or films, with an exposure time of from five to sixty seconds. Luminol is an extremely sensitive reagent for hematin. Inorganic catalyzers, such as hypochlorites, manganese peroxide, ferri-cyanide, colloidal platinum, osmium tetroxide, and gold chloride do not come into consideration in forensic cases. However, they may all be easily identified by chemical reactions. Other materials and substances in common daily use do not give luminescence.

#### REFERENCES

1. Gleu, Karl, and Pfannstiel, Karl. Ueber 3 Aminophthalhydrazid, *J. prakt. Chem.*, N. F. 146, 137, 1936.
2. Wegler, Richard. Chemiluminescenz evchischer Hydrazide. *J. prakt. Chem.*, N. F. 148, 136, 1937.
3. Schmitz, A. J. Diss. Heidelberg, 1902.
4. Curtius, T., and Semper, A. Verhalten des Diäthylesters der 3 Nitrobenzoldicarbon-säure gegen Hydrazin. *Bei.* 46, 1162, 1913.
5. Albrecht, H. O. Über die Chemiluminescenz des Aminophthalhydrazids, *Ztschr. f. physiol. Chem.* 136, 321, 1928.
6. Harvey, N. Luminescence During Electrolisis. *J. Physiol. Chem.* 332, 1456, 1929.
7. Hundress, F. H., Stanley, L. N., and Parker, A. S. The Preparation of 3 Amino phthalhydrazide for Use in Demonstration of Chemiluminescence, *J. Am. Chem. Soc.* 56, 241, 1934.
8. Harris, L., and Parker, A. S. The Chemiluminescence of 3 Amino phthalhydrazide, *J. Am. Chem. Soc.* 57, 1939, 1943.
9. Specht, W. Die Chemiluminescenz des Hämins ein Hilfsmittel zur Auffindung und Erkennung forensisch wichtiger Blutspuren. *Deutsche Ztschr. f. d. ges. gerichtl. Med.* 28, 225, 1937.

This jar will operate very satisfactorily with illuminating gas, if the method described by Brewer and Brown<sup>5</sup> is followed. The use of this lid with illuminating gas simplifies the use of anaerobe jars, for it eliminates the hydrogen cylinder, reducing valve, generator, and lamp bank or rheostat, thereby reducing the cost of equipment to less than one-half.

#### SUMMARY

The advantages of the Brown anaerobe jar over jars of similar type have been pointed out and a modification has been described which possesses the following additional features: The electrical current does not pass through the jar, thereby eliminating the possibility of explosion due to sparking at loose or broken connections. No rheostat or lamp bank is required; the jar connects directly to the 110 volt current. The general design of the lid has been changed to conserve space and to aid in handling of the equipment.

#### REFERENCES

1. Laidlaw, P. P.: Some Simple Anaerobic Methods, *Brit. M. J.* 1: 497, 1915.
2. Brown, J. Howard: An Improved Anaerobe Jar, *J. Exper. Med.* 33: 677, 1921.
3. Brown, J. Howard: Modification of an Improved Anaerobe Jar, *J. Exper. Med.* 35: 467, 1933.
4. Fildes, P., and McIntosh, J.: An Improved Form of McIntosh and Fildes' Anaerobic Jar, *Brit. J. Exper. Path.* 2: 153, 1921.
5. Brewer, J. H., and Brown, J. Howard: A Method for Utilizing Illuminating Gas in the Brown, Fildes and McIntosh or Other Anaerobe Jars of the Laidlaw Principle, *J. LAB. & CLIN. MED.* 23: 870, 1938.

## AN OXYGENATOR WITH A LARGE SURFACE-VOLUME RATIO\*

JOHN H. GIBBON, JR., M.D., PHILADELPHIA, PA.

THE most essential part of any apparatus for the perfusion of organs is the mechanism for oxygenating venous blood. In order to introduce an adequate amount of oxygen into a swiftly moving stream of blood, the blood must be spread out into a very thin film. Such a film has been produced in a variety of ways: by centrifugal force, by bubbles, by flow through a hanging cloth curtain, and by passage over glass beads. In general, the difficulties encountered are foaming, frothing, hemolysis from the trauma, and production of vasoconstrictor substances in the blood from mechanical agitation.

Most of the oxygenators which have been described contain a relatively large amount of blood in proportion to the rate of blood flow through the apparatus. To perfuse an entire animal<sup>1</sup> it was desirable to have a method of introducing oxygen which did not require a large volume of blood in the oxygenating apparatus. In other words, it was desirable that the ratio of the surface available for a film of blood to the volume of blood which the apparatus contained should be a large one. In addition to the large surface-

\*From the Surgical Laboratories of the Massachusetts General Hospital and the Harvard Medical School, Boston.

Received for publication, October 13, 1938.

volume ratio it was necessary for the oxygenator to accommodate varying rates of flow (100 to 500 cc per minute) without foaming. It was also essential to make the diffusing surface of the apparatus large enough to introduce sufficient oxygen to maintain life in a small animal.

A number of different types of oxygenators have been described. Hooker<sup>2</sup> in 1915 devised a revolving flat disk onto the center of which a stream of blood was led from above. The revolving disk threw the blood in a thin film against the sides of an inverted glass bell jar. Hooker gave no figures as to the rate of blood flow, volume of blood contained or amount of oxygen introduced per minute by this apparatus. The obvious drawbacks were the production of foam and the difficulty in ensuring the blood would remain in a film on the sides of the bell jar. Also in 1915 Richards and Drinker<sup>3</sup> described a method of oxygenating blood by allowing it to run through a silk curtain exposed to oxygen. As the blood saturated the curtain there were two surfaces exposed to oxygen. The rate of flow was 50 cc per minute and it introduced 3.75 cc of oxygen per minute. In 1922 Drinker, Drinker, and Lund<sup>4</sup> used a Hooker type of oxygenator which accommodated a blood flow of from 10 to 60 cc per minute. Bornstein in 1926 oxygenated blood to perfuse the hind limb of a dog by passing blood through a glass chamber filled with glass beads. Oxygen was blown through this chamber in the direction of the blood flow. Blood flowed through the apparatus at a rate of 200 to 500 cc per minute. In 1928 Bayliss, Lee and Ogden<sup>5</sup> devised an oxygenator consisting of a tier of rotating disks. The blood when thrown to the side of the apparatus from the top disk was again led into the center by a stationary funnel shaped retaining wall which dropped the blood upon the second revolving disk. Blowing a stream of oxygen through the apparatus considerably increased its efficiency. With a tier of five disks the oxygenator held 54 cc and accommodated rates of flow up to 260 cc per minute. The surface area was approximately 1,500 sq cm and it was possible to introduce as much as 27 cc of oxygen per minute into the blood. Evans,<sup>7</sup> using this apparatus, experienced trouble with clotting. In 1932 von Euler and Heymans<sup>8</sup> devised an oxygenator consisting of a large spherical glass bulb into which blood and oxygen were injected under pressure through small tipped openings which were almost in contact with each other. The blood was thus blown in fine droplets against the inside of the spherical glass bulb. This apparatus could accommodate rates of flow up to 250 cc of blood per minute and introduced as much as 20 cc of oxygen per minute. The authors state that with "proper handling" no clotting occurred with defibrinated blood. Slight hemolysis was occasionally present. In 1933 Daly and Thorpe<sup>9</sup> described a modification of the Hooker type of oxygenator. It consisted of three horizontal revolving disks and three tall glass cylinders arranged concentrically. This apparatus had a surface area of approximately 11,000 sq cm, held 300 cc of blood, and introduced about 17 cc of oxygen per minute. Crankshank<sup>10</sup> in 1934 described an oxygenator consisting of a revolving spiral sheet of metal inside a horizontal cylinder. This apparatus had a surface area of approximately 7,500 sq cm, contained 400 cc of blood, and was capable of introducing 34 cc of oxygen per minute.

It seemed to us that another and simpler way of producing a very even thin film of blood would be to direct the blood against the inner surface of a vertical revolving cylinder. The centrifugal force would ensure the maintenance of a thin even film. The blood would be collected at the bottom of the cylinder in a stationary cup, into which the revolving end of the cylinder would extend. The passage of the blood from the lower edge of the revolving cylinder to the walls of the stationary cup had to be accomplished without producing foam or hemolysis from trauma. With such an apparatus the blood would leave the lower edge of the revolving cylinder at a tangent to the cylinder, and when it struck the retaining wall of the stationary cup, its direction would be changed to that of a tangent to the stationary cup. It is obvious that if this change in the direction of the blood as it strikes the retaining sides of the stationary cup is kept minimal, less trauma and foaming will occur. Hence it was desirable that the space between the cup and the revolving cylinder should be as small as possible. It is also obvious that with any given gap between the cylinder and the cup, the change in direction of the blood will vary inversely with the diameter of the cylinder. It was with these principles in mind that the oxygenator here described was designed.

*Apparatus.*—The oxygenator consists of a vertical steel cylinder (Fig. 1*A*), 46 cm. in length, with an internal diameter of 14 cm. Roller bearings (*B*, *B'*) are tapped on both ends of the cylinder. The external surface of the lower end of the cylinder is planed off to a depth of 3 or 4 mm. so as to form a shoulder about 10 cm. above the lower end of the cylinder. In the upright position this shoulder rests upon the inner racer of the lower roller bearing (*B'*) and supports the weight of the cylinder (this detail is not indicated in Fig. 1). The cylinder is maintained in its upright position by three square upright pillars of steel (*C* is one of these pillars) firmly attached to a broad steel base (*D*). The outer racer of the lower roller bearing (*B'*) rests upon three blocks of steel (*E*), one projecting from the inner surface of each square upright column. The upper ends of the steel pillars are rigidly held by a flat piece of steel in the shape of a "Y" (*F*). Wing-tipped screws (*G*) pass through holes near the ends of the three arms of the "Y" into the upper end of each upright column. Two screws (*H*) pass through each upright column and impinge upon the outer racers of the upper and lower roller bearings. These six screws prevent rotation occurring in the outer racers and hold the cylinder firmly in position.

The greater part of the space within the revolving cylinder is occupied by a smaller stationary cylinder (*I*). This cylinder (*I*) is hollow and closed at both ends except for a metal tube (*J*) which extends through the cylinder from one end to the other. The upper portion of the tube (*J*) passes through a hole in the center of the heavy metal "Y" (*F*). This tube serves to convey oxygen to the bottom of the apparatus. The oxygen then diffuses upward in close contact with the film of blood and escapes at the top. The cylinder (*I*), by occupying most of the air space within the revolving cylinder (*A*), ensures a smooth upward current of oxygen over the film of blood on the inner surface of the revolving cylinder. The metal plate (*K*) closing the upper end of the cylinder (*I*) extends outward to overlie the upper end of the revolving cylinder

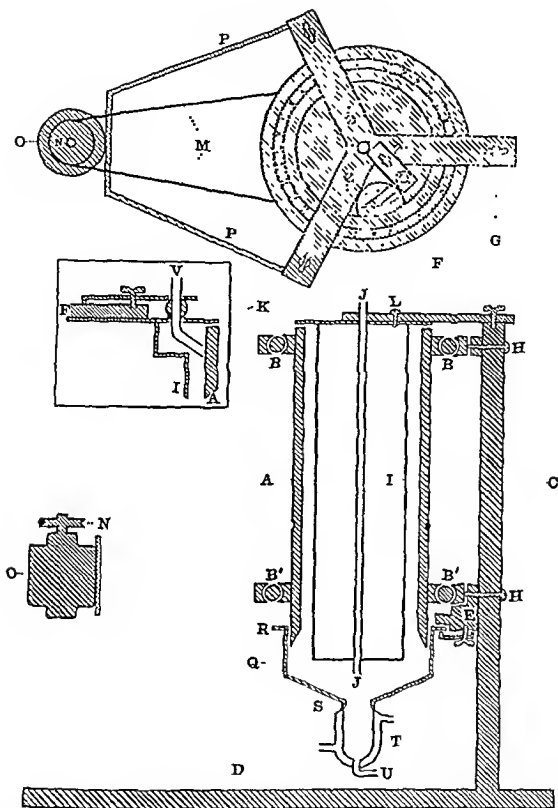


Fig. 1.—A vertical cross section and top view of the oxygenator. The drawing is not made to scale. The insert is a diagrammatic drawing of the tube which conveys the blood to the upper end of the inner surface of the revolving cylinder. For detailed description see text.

(4). The oxygen escapes at the top of the apparatus through the small gap between the metal plate (*K*) and the top of the cylinder (*A*). The central cylinder (*I*) is supported by three screws (*L*) clamping the plate (*K*) to the undersurface of the heavy metal "Y" (*F*).

Revolution of the large external cylinder (*A*) is accomplished by a pulley belt (*M*) passing around the cylinder in a groove on its outer surface. The belt then passes about a small pulley (*N*) fixed on the shaft of a one-sixth horsepower electric motor (*O*) which is mounted vertically. The electric motor (*O*) is supported by steel bars (*P*) projecting from two of the upright steel pillars.



The lower end of the vertical revolving cylinder is tapered from within outward to a sharp edge. Enclosing the lower end of the cylinder (*A*) is a stationary copper cup (*Q*) which receives the blood as it leaves the lower edge of the revolving cylinder. The upper margin of the cup has a small horizontal lip (*R*) extending outward. It is centered exactly in position by blocks (*E*) extending from the three upright columns which come into contact with the outer margin of this lip. The cup is supported in its position by clamping the lip upward against a projecting portion of the steel blocks (*E*).

The walls of the cup (*Q*) in its upper portion are at first vertical and then slope sharply inward to a central hole in the bottom, 3 cm. in diameter. From the undersurface of this hole there extends downward for about 2 cm. a small collar (*S*) with a one to ten taper. A double walled glass cup (*T*) has a glass collar with a similar taper at its upper end. This slips smoothly over the collar at the bottom of the stationary cup and is securely fixed there with deKhotinsky's cement. The glass cup receives the blood descending to it through the oxygenator, and the blood is led out from the bottom through a glass tube (*U*). The cup (*T*) is made of glass to permit observation of the level of the blood within it, and has a double wall through which warm water is circulated.

The blood enters the oxygenator at the top through a curved tube (*V*, insert Fig. 1) which pierces the top (*K*) of the stationary cylinder (*I*) and approaches the inner surface of the revolving cylinder (*A*) at an acute angle (top view, Fig. 1). The blood is thus directed against the upper part of the inner surface of the revolving cylinder with relatively little change in direction. This is an important consideration in the avoidance of splashing and foaming. The details of clamping the tube (*V*) so as to permit adjustment in different directions can be seen in the insert at the left in Fig. 1, and in the view from above at the top. In the latter view the semicircular window cut in the top (*K*) of the cylinder (*I*) can be seen. This window permits adjustment of the tube (*V*) under direct vision. The window is closed when blood is being oxygenated to ensure an even outward flow of oxygen as it escapes between the plate (*K*) and the upper end of the revolving cylinder.

Lamb's blood was used to test the efficiency of the apparatus. The blood was obtained from an abattoir and kept in an icebox when not in use. No observations were made with blood more than forty-eight hours old. There was always some hemolysis present in the blood obtained from the abattoir. The hemolysis was slightly more pronounced after prolonged passage, one hour or more, of the blood through the oxygenator.

The observations were made as follows: Pure nitrogen was blown through the gas intake tube of the oxygenator. The blood to be used was passed through the revolving oxygenator for from forty to sixty minutes, until it became definitely venous in color. It was collected under oil and a sample was withdrawn for determination of oxygen content and capacity. A two liter flask full of this blood, still protected from contact with air by a layer of oil upon its surface, was then passed through the oxygenator while a mixture of 95 per cent oxygen and 5 per cent carbon dioxide was blown through the gas inlet tube. Five per cent carbon dioxide was used because

it roughly approximated the carbon dioxide content of the alveolar air and would thus maintain the carbon dioxide content of the blood at an appropriate level during perfusion experiments.

The blood was led from the glass cup at the bottom of the oxygenator into a second flask and collected under oil. The passage through the oxygenator was started and stopped abruptly, and was timed with a stop watch. The total amount of blood passed through in this time was measured. A sample was then taken of the blood and the oxygen content and capacity were determined.

TABLE I

OBSERVATION NO	BLOOD SAMPLE	OXYGEN CAPACITY	OXYGEN CONTENT	SATURATION	OXYGENATOR RATE	BLOOD FLOW	OXYGEN INTAKE	OXYGEN INTAKE PER CC BLOOD
		vol %	vol %	%	l/m	cc/m	cc/m	cc/m
1	First	20.75	16.56	80.0	11.5	253	10.3	0.0407
	Second	21.00	20.65	98.0				
2	First	17.45	12.5	78.0	12	34	18.0	0.037
	Second	17.20	16.90	98.0				
3	First	16.76	10.60	63.0	14	48	14.0	0.0285
	Second	16.05	11.46	84.0				
4	First	15.95	12.20	76.0	41	408	7.4	0.0160
	Second	16.05	13.82	86.0				
5	First	16.56	11.96	72.0	42	51	14.0	0.0264
	Second	16.18	14.0	90.0				
6	First	17.00	11.83	69.0	419	92	8.6	0.0219
	Second		14.02	83.0				
7	First	18.30	9.29	51.0	491	465	9.7	0.0209
	Second	17.7	11.38	65.0				
8	First	17.37	11.8	68.0	489	504	12.5	0.0248
	Second	17.50	13.87	79.0				
9	First	17.85	15.42	86.4	450	251	15.1	0.0601
	Second	18.15	11.45	63.0				
10	First	18.05	3.57	19.8	251	237	9.5	0.0401
	Second	17.85	7.0	42.6				
11	First	17.42	3.68	21.1	150	222	5.39	0.0243
	Second	17.50	6.11	34.9				

The efficiency of the oxygenator in introducing oxygen into venous blood can be seen in Table I. The rate of revolution of the cylinder, the blood flow, and the oxygen saturation of the venous blood varied in different observations. The oxygen intake per minute was from 5.39 to 18.0 cc. The oxygen intake per cubic centimeter of blood per minute varied from 0.0160 to 0.0601 cc. The surface area of that portion of the apparatus which is covered by a film of blood is 2300 sq. cm. Varying the flow of blood through the apparatus from 50 to 600 cc per minute does not result in forming a reservoir of 15 cc of blood was maintained in the glass cup at the bottom of the oxygenator in order to avoid the possibility of air being drawn into the artery of the animal to be perfused. Aside from this reservoir, 35 cc of blood are contained upon the filming surfaces during operation of the apparatus.

By rough calculations the oxygenator of Bayliss, Fee, and Ogden has a surface-volume ratio of 28; the oxygenator of Daly and Thorpe, a ratio of 37; and the oxygenator of Cruikshank, a ratio of 30. The oxygenator here described has a surface-volume ratio of 66.

#### SUMMARY

An oxygenator with a large surface-volume ratio has been described. It is capable of introducing as much as 18 c.c. of oxygen per minute and accommodates rates of flow of from 50 to 600 c.c. per minute without foaming. It has been used satisfactorily in a large number of perfusion experiments for a period of more than two years, and is thought to be a simple and convenient method of introducing oxygen into blood for perfusion purposes.

#### REFERENCES

1. Gibbon, J. H., Jr.: Artificial Maintenance of Circulation During Experimental Occlusion of Pulmonary Artery, *Arch. Surg.* 34: 1105, 1937.
2. Hooker, D. R.: The Perfusion of the Mammalian Medulla: The Effect of Calcium and of Potassium on the Respiratory and Cardiac Centers, *Am. J. Physiol.* 38: 200, 1915.
3. Richards, A. N., and Drinker, C. K.: An Apparatus for the Perfusion of Isolated Organs, *J. Pharmacol. & Exper. Therap.* 7: 467, 1915.
4. Drinker, C. K., Drinker, K. R., and Lund, C. C.: The Circulation in the Mammalian Bone Marrow, *Am J. Physiol.* 62: 1, 1922.
5. Bornstein, A.: Über Durchblutungsversuche an der überlebenden Hundestremität, *Arch. f. exper. Path. u. Pharmacol.* 115: 367, 1926.
6. Bayliss, L. E., Fee, A. R., and Ogden, E.: A Method of Oxygenating Blood, *J. Physiol.* 66: 443, 1928.
7. Evans, C. L., Grande, F., and Hsu, F. Y.: Two Simple Circuits for Blood-Fed Hearts, *Quart. J. Exper. Physiol.* 24: 283, 1934.
8. von Euler, U. S., and Heymans, C.: An Oxygenator for Perfusion Experiments, *J. Physiol.* 74: (Proc.) 2, 1932.
9. Daly, I. de B., and Thorpe, W. V.: An Isolated Mammalian Heart Preparation Capable of Performing Work for Prolonged Periods, *J. Physiol.* 79: 199, 1933.
10. Cruikshank, E. W. H.: A Magnetic Blood Oxygenator, *J. Physiol.* 82: 26, 1934.

### DETERMINATION OF SULFANILAMIDE IN TISSUE, URINE, AND BLOOD\*

#### A MODIFICATION OF MARSHALL'S METHOD

FRANK T. MAHER, M.S., AND W. J. R. CAMP, M.D., PH.D., CHICAGO, ILL.

**I**N A STUDY of the distribution of sulfanilamide in the body two difficulties were met in using Marshall's original method<sup>1-4</sup> for the determination of this substance: (1) a brown interfering color developed, and (2) quantitative recovery of the drug added to tissue and fluid samples before extraction was not always possible.

\*From the Laboratory of Pharmacology and Therapeutics, University of Illinois, College of Medicine, Chicago.

Received for publication, October 11, 1938.

No criticism is offered here of Marshall's improved method for the determination of sulfanilamide.<sup>4</sup> However, the subjoined methods are capable of returning a high degree of accuracy, and are convenient in application and simple in technique.

In determining the effect of hydrochloric acid on sulfanilamide it was noted that in the solution neutralized with sodium hydroxide the brown color developed. Sulfanilamide solutions containing 0.25 to 1.5 per cent sodium chloride also developed this color when analyzed.

Standard solutions of sulfanilamide containing excesses of silver nitrate from 1 to 5 per cent were repeatedly compared to standard solutions of sulfanilamide in distilled water alone, and the results of these trials uniformly indicate that these excesses of silver nitrate have no detectable effect on the determination of the drug. Hence this substance is used to remove the chlorides.

#### *Materials Used*

- 1 Alcohol, U.S.P., 95 per cent
- 2 Aqueous solution of silver nitrate 20 per cent weight per volume
- 3 Aqueous solution of para-toluenesulfonic acid 20 per cent weight per volume
- 4 Freshly prepared 0.1 per cent aqueous solution of sodium nitrite
- 5 Alcoholic solution of dimethylamino alpha-naphthylamine, 0.4 per cent
- 6 Appropriate standard solutions of sulfanilamide
  - (a) Aqueous solutions, 1 mg per cent and 2 mg per cent sulfanilamide
  - (b) Alcoholic solutions, 1 mg per cent and 2 mg per cent sulfanilamide

Standard solutions can be prepared from stock solutions, 100 to 400 mg per cent, which keep well if stoppered and kept from light. Fresh dilute standards should be prepared about every three days.

*Extraction From and Determination in Tissue*—Twenty to 40 gm samples are ground with clean dry sand, dried on a steam bath and ground to a uniform powder, which is packed into a U.S.P. type percolator and washed thoroughly with petroleum benzene the excess of which is removed by suction until the column of powder is dry. This procedure obviates the loss of material by removal from the percolator. The fat-free residue is macerated for twelve hours with 95 per cent alcohol and then percolated, a total of 500 cc of percolate being collected.

To 5 cc of the well mixed percolate, and simultaneously to 5 cc of an appropriate alcoholic standard, are added 5 cc of water and 1 cc of 20 per cent silver nitrate solution. After standing five minutes, 1 cc of 20 per cent para-toluenesulfonic acid solution is added, followed by 1 cc of freshly prepared 0.1 per cent solution of sodium nitrite. Five minutes later, 5 cc of the 1:250 solution of dimethylamino alpha-naphthylamine in alcohol are added. This mixture is agitated, allowed to stand ten to fifteen minutes, and then filtered until clear, using talc if necessary. The sample and standard solutions so prepared are then compared in a colorimeter.

In order to check the accuracy of the method as outlined for tissue, samples of 20 to 40 gm (moist) of tissue, taken from the brain, heart, liver, spleen, kidneys, lungs, stomach, skin, and muscle, respectively, were ground up as directed with sand, and the mixtures placed in evaporating dishes on

the steam bath. To these moist tissue specimens before drying and extraction, a known volume of standard sulfanilamide solution in alcohol was then added. Upon such prepared, known samples, the process of extraction and determination was then applied as given above, with results as indicated in Table I.

TABLE I

DOG	SULFANILAMIDE ADDED	SULFANILAMIDE RECOVERED WITH $\text{AgNO}_3$	PER CENT RECOVERED
Brain, 20 gm.	10 mg.	10.0 mg.	100.0
Spleen, 25 gm.	10 mg.	10.0 mg.	100.0
Liver, 40 gm.	10 mg.	10.06 mg.	100.6
Heart, 35 gm.	10 mg.	9.8 mg.	98.0
Kidney, 30 gm.	10 mg.	10.12 mg.	101.2
Pancreas, 25 gm.	10 mg.	9.8 mg.	98.0
Muscle, 50 gm.	10 mg.	9.9 mg.	99.0
Skin and hair, 20 gm.	10 mg.	10.2 mg.	102.0

In testing the accuracy of the methods for tissue analysis, the known quantities of sulfanilamide were added to the moist, ground tissue samples before extraction, and not to the extractive liquid prepared from such samples, as was done in previous work.<sup>4</sup> By adding the drug to the moist tissue, the samples, we believe, more nearly resembled unknown tissues brought in for analysis, and offered a test of the efficiency both of the extractive and of the determinative processes.

Two mice injected intraperitoneally with 4 mg. and 3.6 mg. of sulfanilamide, respectively, were placed in beakers for one hour. They were then killed, ground, and prepared as a sample by the above method, adding to the sample the washings from the beakers used to house the animals. The process of extraction and determination, applied to these samples, gave the following results:

No. 1. Free sulfanilamide, 98 per cent; total sulfanilamide, 100 per cent.

No. 2. Free sulfanilamide, 96.7 per cent; total sulfanilamide, 103.4 per cent.

Unknown samples were prepared by one worker and analyzed by another with consistent recovery of  $\pm 3$  per cent.

*Determination of Sulfanilamide in Urine.*—Urine is diluted 1:10 or 1:20 for comparison with a 1 mg. standard.

To 10 c.c. of such dilution, and simultaneously to 10 c.c. of the appropriate aqueous standard, is added 1 c.c. of 20 per cent silver nitrate solution. After standing five minutes, 1 c.c. of para-toluenesulfonic acid solution, 1 c.c. of sodium nitrite solution, and 5 c.c. of 1:250 alcoholic solution of dimethylamino-alpha-naphthylamine are added to each solution. Ten to fifteen minutes later the mixtures are filtered until perfectly clear, using tale if necessary, and the sample and the standard solutions so prepared are then compared in a colorimeter. The results here obtained represent the amount of the drug in the free state.

We have been able to verify the statement that in man and in various lower animals, excepting the dog, sulfanilamide is excreted in a free and a combined form. To determine the total amount excreted, 10 c.c. of diluted

nine and 10 cc of aqueous standard are treated separately with 1 cc of 20 per cent para toluenesulfonic acid in a boiling water bath for ninety minutes. Twenty five cubic centimeter test tubes graduated at 10 cc, were found to be convenient.

The solutions are then cooled, the volume made up to 10 cc with distilled water and then treated as for determination of free sulfanilamide in the urine.

The methods for urine were checked as completely as possible by adding known quantities of sulfanilamide to the urine and proceeding as above. Results within  $\pm 3$  per cent theoretical content were consistently reported. Color matches were satisfactory if silver nitrate was used, the omission of the silver nitrate resulted in the production of brownish colors in the samples.

*Determination of Sulfanilamide in Blood*—One volume of fresh or oxalated blood is laked by the addition of seven volumes of water, about ten to fifteen minutes being allowed for the laking. Two volumes of para toluenesulfonic acid solution are added, and the mixture is thoroughly shaken. After about five minutes, the mixture is poured upon a filter of a size just capable of holding the entire volume of the mixture. The filtrate is received in a volumetric flask or calibrated container marked at the total volume of the precipitation mixture, or ten times the volume of blood taken.

When the liquid has completely drained from the filter the original flask is washed with a small volume of water and this water poured over the precipitate on the filter. This is repeated and the residue on the filter is thus carefully washed with small portions of water until the filtrate reaches the mark on the receiving flask.

To 10 cc of this acid filtrate, 2 cc of 20 per cent silver nitrate solution are added. After about five minutes 1 cc of sodium nitrite solution and 5 cc of dimethylamino alpha naphthylamine solution are added (total volume here 18 cc). At the same time to 10 cc of the appropriate aqueous standard, 1 cc of silver nitrate solution is added after five minutes 1 cc of para toluenesulfonic acid solution 1 cc of sodium nitrite solution, and 5 cc of the dimethylamino alpha naphthylamine solution are added (total volume 18 cc). After standing ten to fifteen minutes the solutions are filtered until clear, using talc if necessary and the clear solutions are then compared in a colorimeter. As was the case with the urine determination this result expressed the blood content of free sulfanilamide. To determine the total drug contained in the blood sample, hydrolyze the 10 cc of the above acid filtrate. After such hydrolysis, results obtained by the determination method express the total sulfanilamide contained in the blood under examination.

In this method as stated for blood we have eliminated the use of acid standard solutions, because of the apparent instability of such acid standards upon standing—thus eliminating what may well be in routine laboratory application, a source of appreciable error. Moreover the accuracy attending the use of solutions acidified as above at the time of the determination has been uniformly established throughout the series of experiments upon blood samples.

The accuracy of the method given here as applied to blood was checked by taking blood samples from a series of dogs laking them as directed with

water, but adding to the laking water known quantities of sulfanilamide. Precipitation, filtration, and determination were then carried out upon the samples so prepared, and results within 2 per cent of the amount added to the samples were uniformly recorded. Again in the case of blood it was repeatedly shown that the addition of silver nitrate solution was necessary for the removal of materials interfering with the formation of the color desired for the assay.

TABLE II  
RESULTS ON A SERIES OF PREPARED BLOOD SAMPLES (DOG BLOOD)

SAMPLE	SULFANILAMIDE ADDED	SULFANILAMIDE RECOVERED WITH $\text{AgNO}_3$	PER CENT RECOVERED
Citrated blood, 10 c.c.	1 mg.	1.0 mg.	100.0
Oxalated blood, 10 c.c.	1 mg.	1.0 mg.	100.0
Oxalated blood, 10 c.c.	2 mg.	1.96 mg.	98.0
Fresh blood, 10 c.c.	1 mg.	0.997 mg.	99.7
Fresh blood, 10 c.c.	2 mg.	2.03 mg.	101.5

The method presented here was applied to samples obtained by adding known quantities of sulfanilamide to water used in laking blood, prior to precipitation and filtration, and hence indicates the efficiency of the process through the various stages.

*Interfering Substances Other Than Chlorides.*—In one case blood samples were taken from a dog after the dog had received several injections of adrenalin. These samples, after the addition of sulfanilamide and the application of the process given for blood, gave results nearly double the actual content of sulfanilamide. It was also known that aqueous solutions of adrenalin alone, when treated by the process given for the determination of sulfanilamide, gave a pronounced dark red color, capable of seriously clouding the accuracy of any determination of sulfanilamide color. Ephedrine was shown to have a similar reaction, and coupling colors were subsequently demonstrated with a number of amino compounds, notably guanidine, and many of the amino acids. These results indicate that sulfanilamide determinations made upon the blood samples of a patient may be influenced by other substances given the patient simultaneously with the sulfanilamide.

These substances are not removed by silver nitrate.

#### DISCUSSION

The fact that the addition of chlorides to a sulfanilamide solution intensifies the interfering color, while the use of silver nitrate removes it, indicates that the chlorides present in the material are responsible in large part for the difficulties encountered. Apparently the silver nitrate removes other substances as judged from the muddy brown precipitate.

In the determinations made upon blood samples, it was noted that 1 c.c. of silver nitrate solution was not sufficient to remove all the interference, although that quantity had been sufficient for the samples from tissue and urine. Increasing the quantity of silver nitrate to 2 c.c. effectively removed interferences.

## CONCLUSIONS

- 1 A modification of Marshall's method for the determination of sulfanilamide in tissue, urine, and blood is given
- 2 Silver nitrate is used to remove interfering substances, notably chloride. An excess does not interfere with the determination
- 3 Consistent results within  $\pm 3$  per cent are obtained
- 4 Epinephrine, ephedrine, guanidine and other amino compounds interfere with this test

We wish to thank the Department of Medical Research Winthrop Chemical Co. Inc. for the sulfanilamide used in these experiments

## REFERENCES

- 1 Marshall, E. K. Determination of Sulfanilamide in Blood and Urine, *J. Biol. Chem.* 122: 263, 1937
- 2 Marshall, E. K. Determination of Sulfanilamide in Blood and Urine, *Proc. Soc. Exper. Biol. & Med.* 36: 422, 1937
- 3 Marshall, E. K., Cutting, W. C., and Emerson, K., Jr. Acetylation of Para-amino benzenesulfonamide in the Animal Organism, *Science* 85: 202, 1937
- 4 Marshall, E. K., Emerson, K., Jr., and Cutting, W. C. The Distribution of Sulfanilamide in the Organism, *J. Pharmacol. & Exper. Therap.* 61: 196, 1937
- 5 Marshall, E. K. and Litchfield, J. T. The Determination of Sulfanilamide, *Science* 88: 2273, 1938

## THE OPTIMUM DOSE OF SULFANILAMIDE FOR GUINEA PIGS\*

ARTHUR RILEY ARMSTRONG, B.S., M.D., and J. LONDON THOMPSON  
ONTARIO, CANADA

WITH THE TECHNICAL ASSISTANCE OF AMELIA RUTH THOMAS, M.T.

THE optimum dosage of sulfanilamide necessary to combat infection in human beings effectively has been established fairly well. That amount which will maintain a free sulfanilamide level of 7 to 10 mg. per 100 c.c. of blood is considered requisite, except in genitourinary disease. To accomplish this between 50 and 100 grams must be given daily, in divided doses. We, however, could find no information in the literature as to the amount of sulfanilamide required to maintain a similar blood level in guinea pigs. Investigators evidently have employed an empirical dosage in their experimental work. The following investigation was conducted, therefore, with a view to obtaining information on this question. It was felt that publication of the findings might be of value to others engaged in sulfanilamide studies.

## EXPERIMENTAL

The animals used were guinea pigs, approximately 12 months old, weighing on an average 620 gm. A total of 150 pigs were used in preliminary tests.

The mode of administration of sulfanilamide was by stomach tube. A No. 7 French catheter was used for this purpose. A 10 per cent suspension of the drug was delivered into the tube by syringe through a No. 21 gauge needle.

\*From the Department of Pathology, Mountain Sanatorium, Hamilton, Ontario.  
Received for publication October 21, 1938.



Suspensions of sulfanilamide sufficiently fine to pass through a No. 21 gauge needle can be made as follows: A 10 per cent hot solution is rapidly cooled to slightly below room temperature under a stream of cold water. As soon as crystals begin to form, the solution is shaken until it is cooled. In the event the suspension is not fine enough, the process is repeated, using more rapid cooling and more vigorous shaking.

Free sulfanilamide determinations were made on blood, using Marshall's method.<sup>1</sup> Owing to the difficulty of obtaining blood from guinea pigs, an animal was killed for each determination and bled from the jugular vein. In a few cases blood was obtained from the heart once, and sometimes twice, but thereafter the animals were killed for the third specimen. With very few exceptions 3 animals were tested for each period observed.

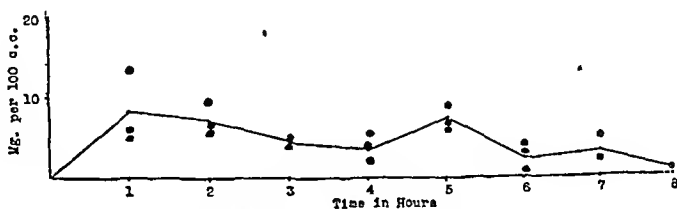


Fig. 1.—Blood sulfanilamide after a single 200 mg. dose.

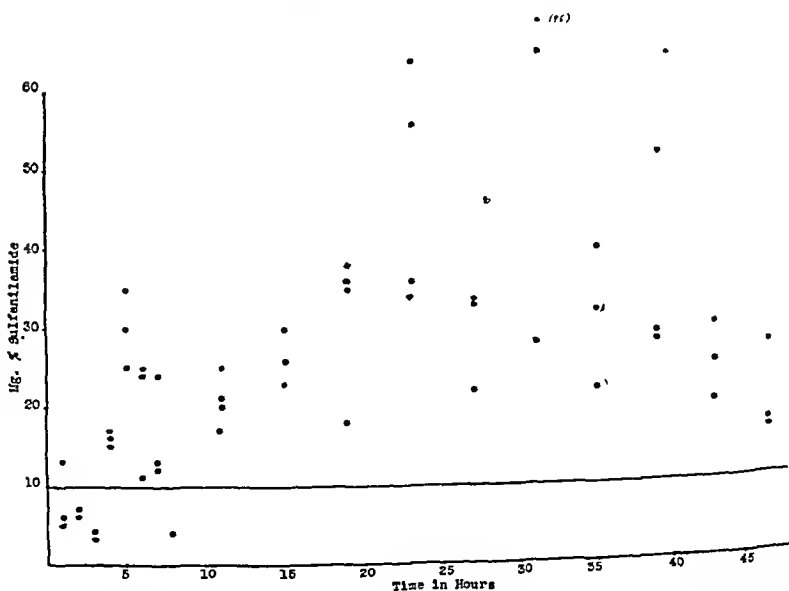


Fig. 2.—Blood sulfanilamide after repeated 200 mg. doses. Two hundred milligrams of the drug were administered at the following intervals: 0, 3, 7, 11, 15, 19, 27, 35, and 43 hours.

Fig. 1 shows the effect of a single 200 mg. dose. Throughout an eight-hour period, during which blood was analyzed each hour, all but one test revealed a value below 10 mg. per 100 c.c.

Fig. 2 illustrates the free blood sulfanilamide levels found when a 200 mg. dose was repeated after three hours, and again at four-hour intervals until nineteen hours had elapsed, following which 200 mg. were given at the end of every eight hours. Sulfanilamide determinations made every four hours

reveal that after the first eight hours the values were always greater than 10 mg per 100 cc up to the end of the experiment. Very high values (over 50 mg per 100 cc) were occasionally discovered.

Signs of toxicity were sometimes present at the twenty-third hour, ruffled coats, loss of appetite, slight exanthesis. These disappeared when the animals were put on the eight hour dose regime.

#### COMMENT

It is felt that a sufficient number of pigs were used in the above experiments to permit the conclusion that 200 mg doses given at intervals as described, can be relied upon to elevate and maintain the free blood sulfanilamide to a level exceeding 10 mg per 100 cc in all animals of the same approximate age and weight as those used in the foregoing experiments.

The sulfanilamide used in these experiments was obtained through Dr. Arnold Branch of Ayerst McKenna and Harrison Ltd., Montreal.

The authors wish to express their gratitude to Dr. W. S. Stanbury for valuable advice and criticism.

#### LITERATURE

1. Marshall, E. K., Jr. Determination of Sulfanilamide in blood and urine. *J. Biol. Chem.* 122: 263, 1937.

## A MICROMETER CURVE MEASURING APPARATUS\*

J. A. E. EASTER, M.D., and J. S. HIPPLE, MADISON, WIS.

THE problem of accurate measurement of recorded curves is a frequent one in a physiologic laboratory. The measurements most frequently required are time intervals, involving distance measurements in the horizontal, and wave amplitudes, involving distance measurements in the vertical direction. The accuracy of measurements depends in general on two things: (a) the sharpness of the onset of waves and peaks in the recorded curve and (b) the accuracy of the measuring apparatus. Aside from these factors, the range of variation in the physiologic response of a single feature must be considered when one curve, or one part of a curve, is to be compared with another. This, of course, is a factor which cannot be controlled instrumentally, but must be estimated by multiplying the number of separate observations.

A micrometer measuring device that was constructed in our shops and has proved satisfactory is pictured in Fig. 1. A heavy cast iron base, *a*,  $4\frac{3}{4}$  by  $9\frac{1}{8}$  by  $1\frac{1}{4}$  inches, a second angular piece of cast iron *b*, 3 inches high and  $\frac{1}{2}$  inch thick, attached as shown to the base, and two steel bars, *c* and *d* form the support for the movable carriage and microscope. One of the steel bars, *c*, is  $\frac{1}{2}$  inch square, the other, *d*, is somewhat larger and bent to a V shape at the top. Both bars are  $6\frac{3}{4}$  inches long and are firmly bolted to the upright support *b*. The carriage consists essentially of three parts as follows: (1) Two

\*From the Department of Physiology and Medical School Shops, University of Wisconsin, Madison.

Made possible in part by a grant from the Wisconsin Alumni Research Foundation.

Received for publication October 29, 1938.

plates *c*, each  $3\frac{5}{8}$  by  $1\frac{5}{16}$  by  $\frac{1}{4}$  inches, cut out at each end to fit the steel bars, are attached to a metal plate below. The metal plate has an opening for the microscope. The anterior plate bears a micrometer screw *f*; the posterior plate, two spiral springs *s*, both of which press against the second part of the carriage *g* and serve to move this carriage in a forward and back direction between the limits of the two plates *c*. The anterior plate is provided with a clamp *h* for locking the under framework in any desired position along the steel bars. (2) A brass framework *g* provided with flanges *j* and fitting under the bars. This framework slides freely along the bars under the control of the micrometer head *f* within the limits of the plates *c*. (3) A brass plate *k*, which is fitted in slots in the upper part of the framework (2). This plate bears the microscope and slides laterally under the control of the micrometer head *m* and two spiral springs at the opposite end. The micrometer head and springs are attached to the framework (2).

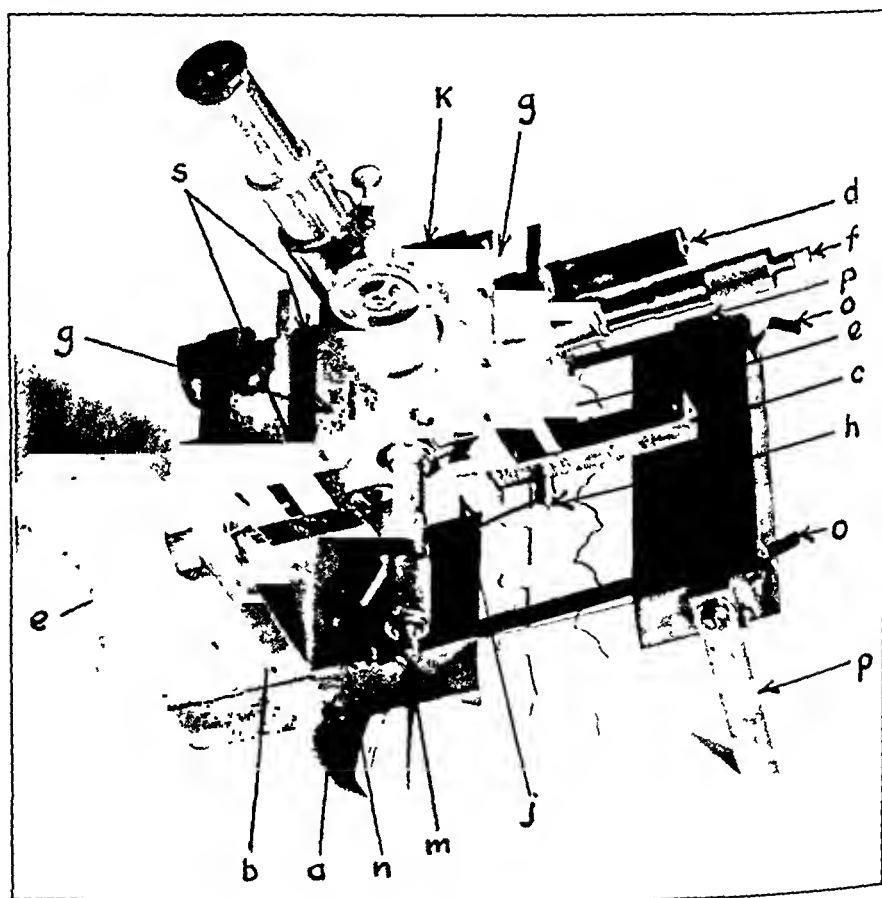


Fig. 1.

A brass plate *n*, the length of which is the same as the width of the base, and which is  $1\frac{1}{2}$  inches wide and  $\frac{3}{16}$  inch thick, is screwed to the base flush with the upright support *b*. This piece bears clips of spring brass *o*, the anterior ends of which clip into recesses cut in the base. The record to be measured is placed on the base with its edge in contact with the plate *n*, covered with a

glass plate and held firmly in place by the spring clips. Two collapsible metal legs *p* tilt the forward part of the base upward to place the microscope at a convenient angle.

The microscope has a magnification of 32 diameters and is provided with cross hairs and a focusing screw. It is model M101A of the Gaertner Scientific Co., Chicago, and was purchased at a cost of \$26.00. The micrometer heads are of the usual type used in machine shops. They have a range of 25 mm and read directly in 0.01 mm divisions.

In using the apparatus the clamp *h* is loosened and the microscope carriage slid forward or backward to cover the portion of the curve to be measured. The clamp is now tightened and vertical measurements for curve amplitude made by the use of micrometer *f*, or horizontal measurements from which to obtain time intervals, made by the use of micrometer *m*. Light reflected from a small table lamp has been found to give sufficient illumination. When measuring curves recorded on transparent film a piece of white paper or thin cardboard is placed back of the film. Illumination by transmitted light can be easily arranged for by cutting out a portion of the base and placing a mirror in a suitable position, but we have not found this to be necessary when tracings or photographic records show a fair degree of contrast.

As an indication of accuracy of measurement the distance between two sharp peaks on two separate curves recorded simultaneously by cathode ray oscillographs was measured fifty times, the cross hairs being moved away before each measurement. The record was made on film at a speed of 211 mm per second. The average time interval between the two peaks in 50 measurements was 0.003465 second. The maximum difference between measurements was 0.000185 second (5.34 per cent of the mean). The standard deviation of the distribution is  $\pm 0.0000548$  second. The coefficient of variation is 1.58 per cent.

---

## A METHOD OF ISOLATING LARVAE OF TRICHINELLA SPIRALIS FOR THE PREPARATION OF THE ANTIGEN USED FOR IMMUNOLOGIC REACTIONS IN TRICHINOSIS\*

---

H. TSUCHIYA, Sc.D., St. Louis, Mo.

---

ATTEMPTS to isolate the larvae of *Trichinella spiralis* free from infected muscles by using artificial gastric juice, as commonly employed in the preparation of the antigen for immunologic tests frequently met with failure. Careful scrutiny of the material thus obtained revealed a few microscopic fragments of undigested muscle fibers. It is highly probable also that a portion of the protein cleavage products resulting from this chemical treatment may remain despite repeated washing of the material. It is generally considered that the presence of host proteins in such preparations may tend to give rise to non-specific reactions in the immunologic tests for the diagnosis of trichinosis.

\*From the Department of Bacteriology, Immunology and Public Health, Washington University School of Medicine, St. Louis.

Received for publication December 3, 1938.

The following method is, therefore, offered as a possible means of eliminating host proteins in the preparation of the antigen used in the tests.

The trichinella larvae are obtained from a rat or guinea pig, about two months old, previously infected with approximately 2,500 larvae isolated from infected muscles by the use of artificial gastric juice, as described by McCoy, Miller, and Friedlander.<sup>1</sup> On the sixteenth to the nineteenth day (the optimum being the seventeenth day) following infection, when the larvae had just threaded their way and come to lie along the long axis of the muscle fibers without definite coiling—the pre-encystment stage, the animal was killed. After the removal of skin, bone, and viscera, the carcass is ground up finely in a meat grinder. The ground material is then placed on two layers of gauze at the bottom of an ordinary potato ricer and enough lukewarm water is added to cover the material. The larvae are then readily forced out of the muscle fibers by gentle squeezing. The expressed juice, consisting chiefly of hemolyzed red blood cells and leucocytes together with many larvae, is collected in a flask. This is now filtered through a standard screen such as is made by the Tyler Company (200 meshes per inch, each opening measuring 0.074 mm.). The majority of viable larvae become coiled up by this treatment, and since the average diameter of these coiled larvae measures approximately 0.15 mm., they will not go through the mesh. The standard screen upon which the larvae are collected is then turned upside down, and the larvae collected thereon are washed down into a tall cylinder with sufficient amount of lukewarm water. This is left standing for a few hours until the larvae settle to the bottom of the cylinder. Baermann's isolation apparatus<sup>2</sup> of hookworm larvae may be also conveniently utilized for this purpose. After repeated washings with lukewarm water, the larvae are finally collected in a test tube and frozen by the use of the lyophile apparatus. The desiccated larvae are then pulverized and extracted with ether for forty-eight hours at room temperature, and then extracted with Coca solution. After centrifuging, the supernatant fluid is drawn off, filtered through a Seitz filter, and stored in ampoules for use as an antigen in immunologic reactions.

#### REFERENCES

1. McCoy, O. R., Miller, J. J., and Friedlander, R. D.: The Use of Intradermal Test in the Diagnosis of Trichinosis, *J. Immunol.* 24: 1, 1933.
2. Baermann, G.: Eine einfache Methode zur Auffindung von *Ankylostomum* (Nematoden) Larven in Erdproben, *Med. Geneesk. Weltevreden, Feestbundel*, pp. 41-47, 1917.

## FREE CHLORINE AS A SOURCE OF ERROR IN BLOOD SUGAR DETERMINATIONS\*

ARTHUR K. ANDERSON, PH D., AND ISADORE ZIPKIN, B A., STATE COLLEGE, PA

RECENTLY we have experienced a difficulty in determining blood sugar by the Fohn and Wu<sup>1</sup> method which we feel should be called to the attention of those working with this or similar methods. The difficulty was a rapid fading of the blue color upon dilution with water before making the colorimeter reading. Within five minutes the solution became colorless. Fading occurred even when a freshly prepared 1:4 dilution of acid molybdate solution was used as the diluting agent. After considerable search for the difficulty, it was finally found that the fading was due to the reoxidation of the blue compound to a colorless compound by free chlorine in the distilled water. The distilled water used was found to contain 0.2 part per million of free chlorine, as measured by the ortho-tolidine method.<sup>2</sup> Another source of distilled water was found to contain 0.1 part per million of free chlorine. This water did not cause complete fading, but fading was noticeable. When chlorine free water was used, no fading was noted, the solutions retaining their blue color overnight.

It is felt that wherever chlorinated water is used in the preparation of distilled water, the danger of free chlorine distilling over is eminent. This is especially true where adequate control of the chlorination process is not maintained.

Although the effect of free chlorine has been noted only in connection with the Fohn and Wu method for determining blood sugar, it is felt that it would interfere with other similar methods for sugar and also with any other method where color is the result of a reduction process.

### REFERENCES

- 1 Fohn, O., and Wu, H. A System of Blood Analysis. Supplement I. A Simplified and Improved Method for Determination of Sugar, *J. Biol. Chem.* 41: 367, 1920.
- 2 Standard Methods of Water Analysis, ed. 8, New York, 1936. American Public Health Association, pp. 19-25.

\*From the Department of Agricultural and Biological Chemistry, the Pennsylvania State College, State College.

Received for publication November 1, 1938.

## A METHOD OF PANCREATECTOMY IN PIGEONS\*

J. M. JANES, B.A., M.D., TORONTO, CANADA

KREBS† has used the breast muscle of depancreatized pigeons as a test object in the study of the influence of added insulin upon the intermediate products of carbohydrate metabolism. Great difficulty was encountered in these studies due to the high mortality attendant upon the removal of the duodenal loop containing the pancreas as well as the inability of maintaining these birds alive longer than three to four days.

Following Krebs' work, a study was made to find an adequate method of pancreatectomy in pigeons. The following is a brief description of the method finally adopted:

The pigeon is tied to a board by its feet and wings. It is given an ether anesthetic by means of dropping ether on a towel folded around its beak and head. The feathers are then plucked from its breast and abdomen. A curved incision, immediately below and parallel to the breast bone, is made through the muscle into the peritoneal cavity on the right side. The duodenal loop, which extends into the left side of the peritoneal cavity, can be recognized by its greater diameter, as compared to the rest of the bowel, and by the presence of the pancreas lying in the loop on both its ventral and dorsal aspects. The color of the pancreas varies from a rather bright red to a light tan. The distal portion of the duodenal loop is grasped in one hand with a square of surgical gauze soaked in saline. With a similar piece of gauze in the other hand, the portion of pancreas on the ventral surface of the loop is gently stripped from the mesentery on which it lies. It is necessary to exercise great care in securing the proper cleavage plane in order not to damage the blood supply to the adjacent bowel. Four of our series of 24 pigeons died as a result of such damage. In stripping off the pancreas it is necessary to maintain pressure on the bleeding points with the saline-soaked gauze. The large segment of pancreas on the ventral side strips quite readily, but the small portion, located at the proximal end of the loop, is removed only with difficulty. It is usually necessary to remove this piece of pancreas with a pair of fine forceps. When working in the proximal region of the loop, care has to be taken not to damage the bile duct which enters the descending limb of the duodenum near the small section of pancreas just mentioned. The portion of pancreas on the dorsal aspect of the loop is stripped off in the manner already described, care being taken as before not to damage the mesentery

\*From the Department of Medical Research, Banting Institute, University of Toronto.

†Professor H. A. Krebs, of Sheffield University, was a guest worker in the Department of Medical Research in April, 1938.

Received for publication, November 3, 1938.



Fig. 1.



Fig. 2.

Fig. 1.—Pancreas in situ in duodenal loop, ventral aspect.

Fig. 2.—Pancreas in situ in duodenal loop, dorsal aspect.



Fig. 3.



Fig. 4.

Fig. 3.—Duodenal loop, eight days after pancreatectomy, ventral aspect. Note increased size of duodenum. Atonic condition probably result of interference of nerve supply.

Fig. 4.—Same as Fig. 3, dorsal aspect.



and to control any bleeding points with gentle pressure of the saline-soaked gauze. After pancreatectomy is complete, the loop is carefully replaced in the peritoneal cavity and closure is effected with white linen sutures.

Prior to pancreatectomy, blood glucose estimations were made, using 0.3 c.c. samples taken from the wing vein. The blood was precipitated by the method of Herbert and Bourne (1930) and true glucose determined with Harding and Downs' (1932) modified Shafer-Hartman reagent. The method of securing the blood is as follows: The feathers are plucked from the axillary region. Using a No. 23 needle, approximately 0.5 c.c. of blood is withdrawn from the wing vein. The blood is transferred to a glass slide, previously coated with paraffin, and then drawn from the slide by means of an 0.3 c.c. pipette. Even though many of the birds were starved for a period of twenty-four hours, it was difficult to secure a true fasting blood sugar on the pigeon because of the retention of food in the crop. Thus preoperative blood sugar levels varied from 124 to 200 mg. per cent. After operation, blood sugars were at first done daily, but it was felt that at least 4 birds died because of too frequent blood withdrawals. Hematomas may be prevented with care.

It is unnecessary to make blood sugar estimations more than once a week following pancreatectomy and even less frequently after the proper dose of insulin has been established. By the fourth day following pancreatectomy, the blood sugar might be expected to be between 200 and 350 mg. per cent. When the blood sugar was under 250 mg. per cent, 0.5 units of insulin (regular) was given daily, but when the values exceeded 250 mg. per cent, 1 unit was given. Without insulin a pigeon will live at least eight days following pancreatectomy. The extreme emaciation noted in depancreatized pigeons after the fourth day can be prevented by the addition of glucose and trypsin to the diet or by giving the birds glucose water to drink.

Out of 24 autopsies done on depancreatized pigeons, a small piece of pancreas was found in only 3 cases. Pigeons prepared and treated in the manner described herein have survived pancreatectomy for over thirty days. Figs. 1 and 2 show the pancreas in situ (ventral and dorsal views). Figs. 3 and 4 show the duodenal loop in a bird killed eight days after pancreatectomy (ventral and dorsal views).

#### SUMMARY

A procedure is described by which pigeons can be depancreatized and maintained for fairly long periods of time.

The morbidity and mortality following such procedure are extremely low.

The use of insulin in the depancreatized pigeon is described.

#### REFERENCES

1. Herbert, F. K., and Bourne, M. C.: The Non-sugar Reducing Substances of Human Blood, With Special Reference to Glutathione, *Biochem. J.* 24: 299, 1930.
2. Harding, V. J., and Downs, C. E.: A Sensitive Copper Solution for Glucose Estimation, *Canad. Chem. Met.* 16: 12, 1932.

## AN ELECTRIC PULSE COUNTER\*

LAURENCE E. MOREHOUSE AND W. W. TUTTLE, IOWA CITY, IOWA

THE desirability for an automatic pulse counter has long been recognized. Although many attempts have been made to develop such equipment, no one has reported a counter which met all the requirements of a practical instrument. These requirements are stability, simplicity of operation, and accuracy.

Recent developments in amplification have made it possible to construct an apparatus that conforms to the requirements of a reliable pulse counter. By use of the instrument herein reported, it is practical to read the heart rate for any interval of time directly from the counter. In addition, the stability of the apparatus assures one of reliable data, even while the person is performing simple types of exercise.

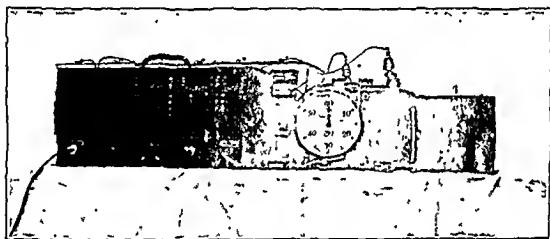


Fig. 1.—The pulse counter.

The principle of the pulse counter is the amplification of the action current produced by each contraction of the heart, sufficient to operate a relay which in turn operates an electromagnetic counter. The device is shown in Fig. 1.

*The Electrodes.*—The electrodes employed are direct-contact electrodes, the same as those used to connect a subject to the electrocardiograph. The position of the electrodes on the body is shown in Fig. 2. In order to insure good contact between the subject and the electrodes, a liberal amount of electrode jelly is employed. The electrodes are held in place by adjustable elastic bands, as shown in Fig. 2.

The input wires are crystal microphone cable, shielded and rubber jacketed. They are of sufficient length to insure free movement of the person during exercise.

\*From the Department of Physiology, State University of Iowa, Iowa City.  
Received for publication, October 27, 1938.

The electrodes offer no contact difficulties if properly connected to the person. If he is at rest, no precautions are necessary. However, if he is to perform exercise, it is well to free the contact surface from hair, fit the electrodes snugly with the elastic bands, and use an abundant amount of electrode jelly under and around the electrodes. Obviously, the lead in wires must make tight connection with the electrodes. Although soldering insures the best contact, the setscrews on the electrodes are adequate for tight connections.

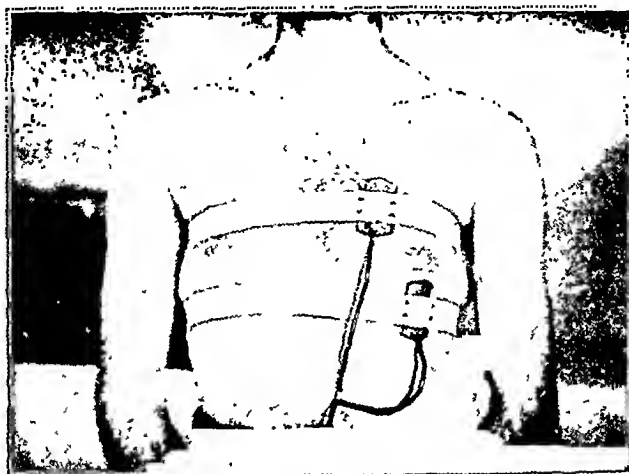


Fig. 2.—The electrodes.

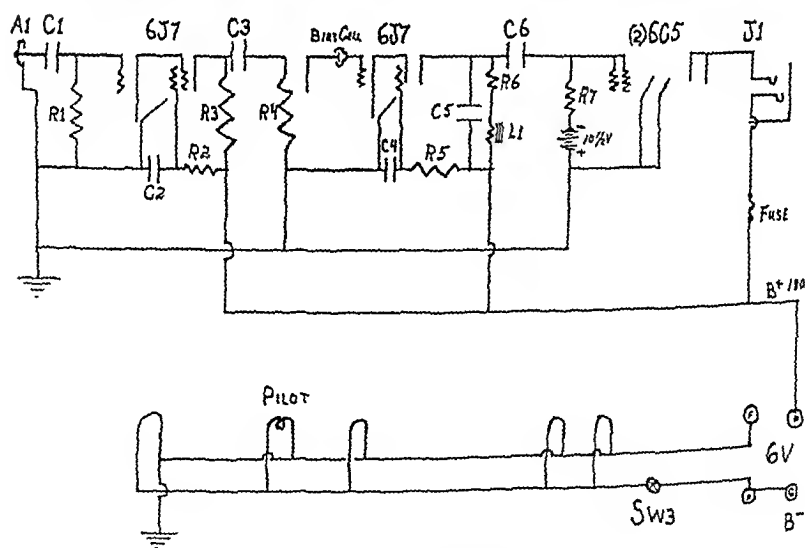


Fig. 3.—Amplifier unit.

R1 IRC BT1 20 meg.  
 R2 IRC BT1 2 meg.  
 R3 IRC BT1 0.5 meg.  
 R4 Yaxley "N" 0.5 meg.  
 R5 IRC BT1 0.5 meg.  
 R6 IRC BT1 9000 ohms.  
 R7 IRC BT1 0.5 meg.  
 C1 Aerovox 684 0.1 mfd.  
 C2 Aerovox 484 0.5 mfd.  
 C3 Aerovox 684 0.5 mfd.

C4 Aerovox 484 0.5 mfd.  
 C5 Aerovox 484 0.1 mfd.  
 C6 Aerovox 684 0.5 mfd.  
 L1 Thordarson T-93C20 250 Heneries.  
 Bias cell—Mallory 1 volt.  
 Fuse—Buss 8 AG 1/4 amp.  
 A1 Amphenol PC1M connector.  
 J1 Yaxley No. 702B jack.  
 SW3 Yaxley No. 10 switch.  
 Pilot Yaxley No. 310R with No. 40 bulb.

*The Amplifier*—The amplifier is a 3 stage high gain impedance resistance coupled, designed to amplify the main peak of the electrocardiogram. The circuits employed are shown in Fig 3. To prevent absorption of energy coming from persons with a high skin resistance, a high impedance input was employed. To this end, a 20 megohm input resistor was used, and the person further isolated from this high resistance by means of blocking condenser *C1*. The 20 megohm input resistor offers the further advantage that bias on the first tube is obtained by the drop caused by grid current flowing through the resistor. This circuit gives greater gain than can be obtained with other bias systems. The impedance *L1* in the plate circuit of the second tube is resonated at approximately 30 cycles by means of condenser *C5*. This tuned circuit has somewhat the effect of a low pass filter.

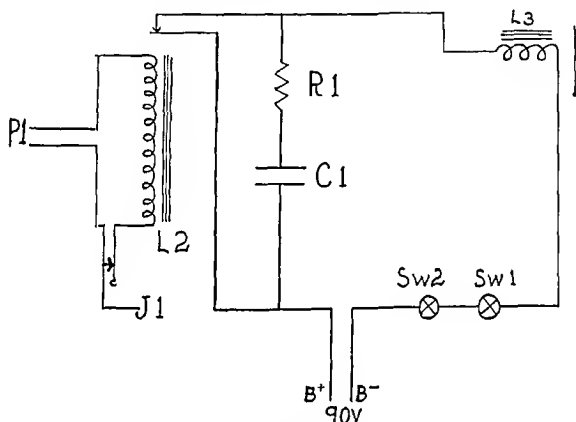


Fig 4—Relay counter unit

P1 Yaxley No 76 phone plug  
L2 Guardian No 5—1000 G I relay  
L3 Western Electric 5AA message register  
R1 IRC BT  $\frac{1}{2}$  500 ohms

C1 Aerovox 681 0.05 mfd  
J1 Yaxley No 701 jack  
SW1 Bud No 743 push button switch  
SW2 Bud No 1003 SPST switch

The output stage consists of two 6C5 tubes in parallel, biased to a "no signal" plate current of about 0.5 Ma. Relay *L2* is activated by action currents passing through the amplifier, causing a change in the plate current as high as 5 to 6 Ma. Precautions have been taken to eliminate the possibility of a shock to the subject by using dissimilar connectors on the input and output circuits.

The relay is contained in the counter unit and is shown in Fig 4.

*The Counter*—The counter employed is a Western Electric message register, which is accurate up to a speed of 300 per minute. The counter unit contains an unusual feature in that the relay circuit contacts are normally closed, the counter being activated on the break rather than on the make, as is found in conventional arrangements. The arrangement of the counter is

shown in Fig. 4. This modification is necessary because of the short duration of the action current produced by the heart. It also eliminates interference caused by sparking of the relay contacts, permitting the apparatus to be operated in an unshielded room. Resistors  $R1$  and  $C1$  are connected across the relay contacts to further reduce any possible sparking. A normally closed push-button switch ( $SW1$ ) has been included in the circuit to enable the counter to be set at a convenient number. A jack ( $J1$ ) has been included in the relay coil circuit to enable a remote control switch or timer to be connected if it is desired.

*Method of Operation.*—The electrodes are fixed in place in the approximate line of the electrical axis of the heart, one over the apex and one over the base, which allows leading off the maximum voltage; the starting switches on the amplifier and relay system are closed, and the gain control on the amplifier is varied until each beat is recorded accurately on the counter. Too low amplification will result in missed beats, and too high amplification will register false beats on the counter. The relay counter unit should not be placed within 5 feet of an a-c circuit as the relay will flutter, giving false counts. Also the relay counter unit must be placed at least 1 foot from the amplifier as the vibrations of the counter may microphonically affect the tubes of the amplifier. Readings of the counter may be taken at desired intervals or the counter may be made to operate during any desired interval which is automatically started and interrupted by a timing device.

#### SUMMARY

An automatic pulse counter which is accurate, stable, and simple to operate, has been devised and described. It is capable of accurate counting even while a person is performing simple exercise. The pulse rate is read directly from the counter, thereby eliminating the objection of recording and then counting.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D. ABSTRACT EDITOR

**ERYTHROBLASTOSIS Foetalis**, Macklin M. T. Lamont J. A. and Macklin, C. C. *Am J Dis Child* 57 349, 1939

A case of erythroblastosis foetalis accompanied by hemolysis and edema is reported in a newborn girl. There was nothing in the maternal history to explain the condition. Five of the seven pregnancies of the mother ended in miscarriage or premature delivery. It is suggested that the earlier products of conception may also have been examples of abnormal production of blood in which the defect was severe enough to interfere with further development.

It is also suggested that the defect may be one of retardation of blood formation in the fetus and not a persistence of a normal fetal blood picture.

**ALLERGY in Childhood**, Ratner, B. J. *A. M. A.* 111 16, 1938

No dogmatic conclusions can be drawn with respect to the onset and progress of allergic syndromes in childhood. This is due to the fact that our knowledge of the underlying mechanism of allergy is still far from complete.

It is apparent that allergy may start at an extremely early age. The newborn period is usually free, with the rare exception of an occasional instance of passive sensitization acquired in utero.

Eczema is the prevailing allergic syndrome in infants under 1 year of age and foods are the prevailing reacting substance. Though food sensitivities were found in 100 per cent of our patients under 1 year of age, they are nevertheless not always the sole cause of the allergic syndrome. The allergic eczemas in many instances (50 per cent) are shown to be due largely to contact with environmental substances to which these same infants react.

Infants who react to egg and other food proteins which they have never ingested have most probably been actively sensitized in utero. The early allergic manifestations may be invoked by a passage of these substances through the breast milk in infants previously sensitized in utero. On the other hand, such potential sensitivities may cause allergic reactions when these foods are eventually added to the diet in the latter part of the first year. The age of onset of eczema is considerably earlier than that of asthma and, in many instances, it is the forerunner of asthma.

After early infancy, asthma becomes the prevailing allergic syndrome, and wherever there is a multiplicity of syndromes, asthma is generally one of the complicating conditions. Asthma was also the dominant form in the allergic antecedents of the children studied. It appears, therefore, that the lung structure is phylogenetically predisposed to sensitization in the human species as it is in the guinea pig.

Hay fever is not of great significance in early infancy in the middle Atlantic States. It comes to the fore in the latter part of the first decade and becomes a prominent syndrome in the second decade of life.

Urticaria at all times remains sporadic and shows no particular age alignment.

Though food sensitivities play a very important role in the allergy of childhood, inhaleds and contactants play an equally important one. Throughout infancy and childhood reactions to foods and inhaleds and contactants run a parallel course. There are many infants and children who react to pollens who do not suffer from hay fever. The pollens may be contributing factors in the eczema or asthma, or they may indicate potential sensitivities which eventually cause the onset of hay fever.

The preponderance of multiple reactivities would suggest that specific therapeutic measures may fail if all the offending factors are not taken into account.

The onset of allergy can in a measure be prevented through control of the diet of the pregnant woman and of the young infant. Cognizance of the important role that environmental inhalant substances play in earliest infancy must lead to prophylactic measures.

The progress of allergy can be interrupted by early diagnosis of the interrelated syndromes and reduction of contact with inciting substances.

It is true that in the present state of our knowledge it is difficult actively to engage the interest of the physician who is unprepared in training and equipment in this subject, which appears to be complicated by so many factors and which from many reports seems to yield but few satisfactory results.

The time must soon arrive, however, when the salient facts, gleaned from the maze of work that is being carried on, will be separated from the chaff and formulated into broad principles that can be more readily applied to general everyday practice.

**NEOPLASMS, Malignant, of Nasopharynx, Hauser, I. J., and Brownell, D. H. J. A. M. A. 111: 2467, 1938.**

Malignant neoplasms of the nasopharynx often manifest themselves by symptoms which are extranasal in nature.

Cervical swellings, changes in the tympanic membrane, unilateral deafness or stuffy sensation in one ear, pain in the head or throat, diplopia or rectus lateralis paralysis, or unilateral paralysis of any cranial nerves calls for careful examination of the nasopharynx.

All malignant neoplasms arising from the lining epithelium of the nasopharynx appear to be medullary squamous-cell carcinomas. Lympho-epithelioma and transitional-cell carcinoma are believed to be highly undifferentiated forms of squamous-cell carcinoma.

Irradiation is at present the only form of treatment. In spite of poor results, if earlier diagnoses were made, chances of arresting or destroying the neoplasm would be greater.

The nasopharynx should be given careful routine inspection by the otolaryngologist.

**POLYCYTHEMIA, Course of, Rosenthal, N., and Bassen, F. A. Arch. Int. Med. 62: 903, 1938.**

Primary polycythemia is a disease of long duration. Occasionally the early stage may be asymptomatic, but usually it is symptomatic.

After a period of years one or more of a variety of clinical conditions apparently develop, as a result either of the hyperactivity of the leucopoietic and megakaryocytic systems or of exhaustion of the erythropoietic system.

In its terminal stages this disease may become leucemic, thrombocythemic, or anemic, or it may reveal various remarkable combinations of any or all of these phases.

**SEDIMENTATION RATE, and Non-filament, Filament Ratio in Low Grade Chronic Illness, Stiles, M. H. Arch. Int. Med. 63: 664, 1939.**

Practically all of a group of 292 patients with symptoms of low grade chronic disease showed an increase in the nonfilament-filament ratio, while more than 80 per cent showed an increased sedimentation rate.

The sedimentation rate and the nonfilament-filament ratio showed only minor variations when distributed according to age, sex, or diagnosis. When distributed according to intensity of symptoms, they showed a direct relationship to increases in the severity of illness.

These findings should prove useful in following the course of low grade chronic disease and in evaluating treatment.

**PNEUMONIA in Infancy, Sulfapyridine Treatment of, Wilson, A. T. et al. J. A. M. A. 112: 1435, 1939.**

In a study to determine the value of sulfapyridine in the treatment of pneumonia in infants and children, no distinction was made between croupous pneumonia and bronchopneumonia. The authors believe that it is necessary to study a control group

simultaneously with a "sulfapyridine group" and to compare such factors as age, severity of pneumonia, and time at which there is clinical improvement, significant fall in the temperature, and clinical recovery. An analysis of these factors is especially important in a study of pneumonia in early life, since the case fatality rate is so low that it cannot be employed as a criterion for comparison. For example, all the patients in both groups in this study recovered.

Seventy patients with pneumonia, half of whom received sulfapyridine, were observed. Analysis of the characteristics of the disease in the sulfapyridine group and in the control group demonstrated that the two groups were suitable for comparison.

The administration of sulfapyridine apparently shortened the course of the pneumonia by approximately three to four days. By statistical analysis it was demonstrated that the fall in temperature and the clinical recovery were significantly earlier in the sulfapyridine group than in the control group.

The series of cases was too small to allow evaluation of the effect of sulfapyridine in preventing complications of pneumonia.

Two of the patients in the sulfapyridine group manifested a course apparently uninfluenced by the drug; two other patients had a relapse and one had a series of relapses whenever the use of the drug was discontinued. One patient in the control group had a relapse.

The optimum dosage of sulfapyridine needs further study. Observations indicate that a dosage which secures a level of free sulfapyridine in the blood of approximately 4 mg per 100 cc is therapeutically adequate.

There were marked individual variations in the levels of sulfapyridine in the blood obtained with the doses we employed, namely, from 1 to 15 grains (0.06 to 0.1 gm) per pound of body weight each twenty-four hours. To be sure that an adequate dosage is being maintained, it is necessary to examine the blood frequently, that is, at least daily. The use of a photoelectric colorimeter makes it possible to perform determinations on 0.1 cc of capillary blood.

Vomiting and cyanosis were present in about half the patients receiving sulfapyridine. The cyanosis was not sufficient to cause concern in any instance. A number of patients in the control group also manifested cyanosis and vomiting during the acute stage of their pneumonia. Two patients receiving sulfapyridine had cutaneous eruptions. None of the severer drug reactions were encountered.

These preliminary observations on the effect of sulfapyridine on the pneumonias of infancy and childhood and the apparent low toxicity of the drug in the doses employed are sufficiently encouraging to warrant further study.

#### WASSERMANN REACTION Positive in Spirochetal Infections Other Than Syphilis, Murrell, T. W. Arch. Dermat. & Syph. 39: 667, 1939

A case is reported in which an undoubted relationship existed between a positive Wassermann reaction and a spirochetal infection other than syphilis.

It is increasingly evident that the so-called false positive reaction is not a rarity, and if eugenic laws based on serologic reports are to be passed, this fact should be given due consideration. In view of the changing status of such reactions, it may be advisable to abandon the term "false positive" as a part of the syphilologist's nomenclature.

#### PNEUMONIA, Acute, in Infants and Children in Johannesburg, Kark, S. L., and Jaspan, E. South African J. M. Sc. 3: 121, 1939

Seven hundred and eighty-four case records have been examined, of which 548 are those of European children and 236 non-European.

The mortality rate of the non-European children was found to be almost double that of the Europeans. No attempt has been made to discuss the reasons for this difference, as there are far too many essential differences between these groups of peoples which have



not as yet been analyzed. Factors such as nutrition, structure, and mode of living have not been investigated, and it would, therefore, be idle for us to speculate on the various causes of this difference in mortality rate.

A clinical differentiation between the lobar and bronchopneumonic forms of acute pneumonia has been attempted. In both the European and non-European peoples, bronchopneumonia is found to be more common in infancy, whereas in older children the lobar type is more common. Bronchopneumonia appears to be more common in older non-European children than in Europeans. The difference in this series is possibly not significant, but the authors have stressed this aspect of the problem in the hope that other workers will be interested in what might prove to be a very valuable study.

In both groups of peoples the authors have found bronchopneumonia to be a greater cause of death than lobar pneumonia, lobar pneumonia in the European group being a relatively rare cause of death (2.7 per cent). In the non-European group lobar pneumonia caused more deaths, having a mortality rate seven times as high as the European. The non-European mortality rate for bronchopneumonia (50.6 per cent) was 11.3 per cent higher than the European.

The incidence and mortality were found to be highest in infancy in the non-European series as well as the European. At all ages the mortality rate of the non-European was higher than the European.

A slight preponderance of males was noted in the series. There was no significant difference in mortality in the two sexes.

The highest incidence of both lobar and bronchopneumonia was found to occur in the winter months. Variation of mean maximum and mean minimum temperatures from month to month did not appear to have any significant effect on the seasonal incidence.

#### **LEUKEMIA, Phagocytic Activity in, Hirschberg, N. Am. J. M. Sc. 197: 706, 1939.**

Phagocytic studies, sedimentation rates, and bacteriologic studies were made in 20 patients with various types of leucemia.

The number of mature neutrophils in the peripheral blood of leucemic individuals which showed phagocytic activity was greatly decreased from normal, and the phagocytic activity of the existing cells was also greatly decreased.

The sedimentation rate was increased in most of the acute cases but was normal in most of the chronic cases of leucemia.

Significant bacteriologic findings were noted in most of the acute cases.

It seems probable that leucemia is not itself an infectious disease but that infection so often found associated with this disease is superimposed upon the leucemia. This may be due to the decreased resistance of the leucemic individual, as evidenced by the decrease in phagocytic activity of the mature neutrophils.

#### **MENINGITIS, Experimental Type II Pneumococcic, Chemotherapy of, Gross, P., Cooper, F. B., and Lewis, M. Am. J. M. Sc. 197: 609, 1939.**

A cerebrospinal meningitis has been produced in rats with a type II pneumococcus. Sulfanilamide therapy of this disease effected a marked reduction in mortality as well as in incidence and severity of the lesions.

Therapy with 4,4'-di-(acetyl-amino)-diphenylsulfone was less effective against this infection than sulfanilamide.

Sulfanilamide is suggested as an adjuvant to specific serum therapy, or as the primary therapeutic agent when such serum is not available, as a means toward an effectively lowered mortality rate of pneumococcic pneumonia and meningitis in man.

#### **PNEUMONIA, Pneumococcus Type I, Specific Treatment of, Finland, M., and Brown, J. W. Am. J. M. Sc. 197: 151, 1939.**

The death rate among patients with type I pneumococcus pneumonia treated with concentrated type-specific antibody at the Boston City Hospital has been regularly one-half or less that of similar contemporaneous nonserum-treated patients. This has remained true

in recent years in spite of the fact that the proportion of persons treated with serum has increased from 43 to 89 per cent. Of the patients treated with serum before the end of the fourth day, the death rate is less than one third of the nonserum treated mortality.

Bacteremic and nonbacteremic persons are equally influenced. The greatest reduction in death rate occurs in patients under 50 but those of other age groups are probably also beneficially affected by serum treatment. Empyema occurred after serum treatment, chiefly in bacteremic patients, and its frequency was proportional to the delay in beginning treatment.

Type I antipneumococcus sera produced in both horses and rabbits were potent and effective. There are insufficient data to indicate any superiority of the one over the other. There were no immediate allergic type of reactions observed in the rabbit serum recipients, but thermal reactions and serum sickness were somewhat more frequent among them.

*Sulfanilamide* in the small number of cases in which it was used alone or with serum seemed to influence the course of type I pneumococcus pneumonia only slightly, if at all.

#### BRUCELLOSIS Significance of Standard Laboratory Procedures in the Diagnosis of, Menefee E E and Poston M A. *Am J M Sc* 197 646 1939

There appears to be a direct relationship between raw milk consumption and skin reaction to brucellergen.

Skin tests are not without danger since severe local and systemic reactions are not infrequent.

There appears to be little correlation between skin reactivity and measurable antibody content of the serum.

Endermic injection of brucellergen will cause a definite increase of circulating antibodies in allergic patients.

Brucellergen followed by typhoid vaccine will cause a greater and more prolonged increase of circulating antibodies than brucellergen alone.

Any febrile reaction will cause an increase of circulating antibodies.

In survey work a better immunologic picture is obtained by measuring the antibodies at the height of the antibody response to brucellergen.

Negative skin tests, agglutinins and opsonocytophagic reactions do not rule out the possibility of *Brucella* infection.

Positive skin tests, low agglutinins and a moderate opsonocytophagic reaction, even when accompanied by suggestive signs and symptoms do not prove the diagnosis of brucellosis.

#### SYPHILIS, With Special Reference to the Incidence in Relationship to Age Groups and Status of Therapy at the Date of Infection Hansmann G H. *J A M A* 12 1796, 1939

Syphilis was studied in the various age groups in Milwaukee, with special reference to incidence and the consequences of the disease as well as the relationship of the incidence to the development of means for recognition and treatment of the condition.

Dependence was placed on routine admissions, presentation of parents for blood transfusions, and post mortem examinations for reliable sampling of the public in the study.

The high incidence of syphilis which prevails in the age group who acquired their syphilis before the advent of the discovery of the spirochete, the development of important arsenic and bismuth preparations, and the knowledge of the serologic tests, indicate that the ground work has been laid for an imminent spontaneous fall in the incidence of syphilis.

The incidence of syphilis varies from one locality to another, depending on the alertness of the profession, the cooperation of the patient, and the provisions for the care of the indigent, named in the order of their importance.

not as yet been analyzed. Factors such as nutrition, structure, and mode of living have not been investigated, and it would, therefore, be idle for us to speculate on the various causes of this difference in mortality rate.

A clinical differentiation between the lobar and bronchopneumonic forms of acute pneumonia has been attempted. In both the European and non-European peoples, bronchopneumonia is found to be more common in infancy, whereas in older children the lobar type is more common. Bronchopneumonia appears to be more common in older non-European children than in Europeans. The difference in this series is possibly not significant, but the authors have stressed this aspect of the problem in the hope that other workers will be interested in what might prove to be a very valuable study.

In both groups of peoples the authors have found bronchopneumonia to be a greater cause of death than lobar pneumonia, lobar pneumonia in the European group being a relatively rare cause of death (2.7 per cent). In the non-European group lobar pneumonia caused more deaths, having a mortality rate seven times as high as the European. The non-European mortality rate for bronchopneumonia (50.6 per cent) was 11.3 per cent higher than the European.

The incidence and mortality were found to be highest in infancy in the non-European series as well as the European. At all ages the mortality rate of the non-European was higher than the European.

A slight preponderance of males was noted in the series. There was no significant difference in mortality in the two sexes.

The highest incidence of both lobar and bronchopneumonia was found to occur in the winter months. Variation of mean maximum and mean minimum temperatures from month to month did not appear to have any significant effect on the seasonal incidence.

#### **LEUKEMIA, Phagocytic Activity in, Hirschberg, N. Am. J. M. Sc. 197: 706, 1939.**

Phagocytic studies, sedimentation rates, and bacteriologic studies were made in 20 patients with various types of leucemia.

The number of mature neutrophils in the peripheral blood of leucemic individuals which showed phagocytic activity was greatly decreased from normal, and the phagocytic activity of the existing cells was also greatly decreased.

The sedimentation rate was increased in most of the acute cases but was normal in most of the chronic cases of leucemia.

Significant bacteriologic findings were noted in most of the acute cases.

It seems probable that leucemia is not itself an infectious disease but that infection so often found associated with this disease is superimposed upon the leucemia. This may be due to the decreased resistance of the leucemic individual, as evidenced by the decrease in phagocytic activity of the mature neutrophils.

#### **MENINGITIS, Experimental Type II Pneumococcic, Chemotherapy of, Gross, P., Cooper, F. B., and Lewis, M. Am. J. M. Sc. 197: 609, 1939.**

A cerebrospinal meningitis has been produced in rats with a type II pneumococcus. Sulfanilamide therapy of this disease effected a marked reduction in mortality as well as in incidence and severity of the lesions.

Therapy with 4,4'-di-(acetylamino)-diphenylsulfone was less effective against this infection than sulfanilamide.

Sulfanilamide is suggested as an adjuvant to specific serum therapy, or as the primary therapeutic agent when such serum is not available, as a means toward an effectively lowered mortality rate of pneumococcic pneumonia and meningitis in man.

#### **PNEUMONIA, Pneumococcus Type I, Specific Treatment of, Finland, M., and Brown, J. W. Am. J. M. Sc. 197: 151, 1939.**

The death rate among patients with type I pneumococcus pneumonia treated with concentrated type-specific antibody at the Boston City Hospital has been regularly one-half or less that of similar contemporaneous nonserum-treated patients. This has remained true

## REVIEWS

### Alcohol in Moderation and Excess\*

THIS is undoubtedly the best discussion of the subject that has appeared since Emerson's *Alcohol and Man* was published several years ago. It has the advantage of brevity. Written at the request of the Virginia State Legislature for school use, it aroused such antagonism among the prohibitionist forces that it was finally burned not in effigy but in fact, in the furnace of the state capitol building designed by Thomas Jefferson, lover of freedom of speech. The one thousand copies ordered by the legislature were so destroyed before they were even read by the legislators.

This book may be most highly recommended as an unbiased discussion of the pros and cons of the subject.

### Surgical Pathology of the Diseases of the Mouth and Jaws†

THIS is the tenth of a series of monographs by Professor Hertzler and, like its predecessors, may be expected to take its place as an outstanding reference text. He writes with a vigorous and refreshing style upon the less well known from his ample experience. It is to be regretted that, as the author states in his preface, this is the last of the series.

His viewpoint is broad and it is apparent that his experience in tissue pathology is as extensive as his experience in surgery. What he has to say is well worth reading. The book is excellently printed and the illustrations are on a par with the preceding volumes of the series. The photographs and microphotographs are of outstanding excellence and are well reproduced.

This volume is well worth its price and can be highly recommended.

### Pocket Medical Dictionary‡

SO MANY, so rapid, and so inevitable are the changes in medical terminology occurring almost from day to day that a medical dictionary is an essential reference.

This pocket edition, while, of course, not replacing the large and more complete volume, is still a multum in parvo, containing over 40,000 words and many useful tables.

As a convenient and handy reference it can be well recommended. Any book which has gone through seventy-five printings has established itself as a standard text.

### A B C of the Vitamins§

WHILE it is possible that this book is intended primarily for the layman, the physician may profitably add it to his reference library.

Despite the voluminous data, not altogether free from complexity, which has accumulated concerning the vitamins, Mrs. Gregory, by means of cleverly constructed charts, presents their story not only clearly, but in a most interesting manner. Her charts are accurate in their data and present the material in a clear and concentrated way. This book may be enthusiastically commended.

\* *Alcohol in Moderation and Excess. A Study of the Effects of the Use of Alcohol on the Human System.* By J. A. Waddell, M.D., Professor of Pharmacology, Materia Medica and Toxicology, Medical Department, University of Virginia, and H. B. Haag, M.D., Professor of Pharmacology, Medical College of Virginia. Bound in boards and cloth. 184 pages. 25 illustrations. \$1.00. William Byrd Press, Inc., Richmond, Va., 1939.

† *Surgical Pathology of the Diseases of the Mouth and Jaws.* By Arthur C. Hertzler, M.D., Professor of Surgery, University of Kansas. Cloth. 248 pages. 206 illustrations. \$5.00. J. B. Lippincott Company, Philadelphia, Pa.

‡ *Pocket Pronouncing Medical Dictionary.* By George M. Gould, A.M., M.D. Revised by C. V. Brownlow. Leatherette ed. 11. \$2.50. P. Blakiston's Son & Co., Philadelphia, Pa.

§ *A B C of the Vitamins. A Survey in Charts.* By Jennie Gregory, M.S. With a Foreword by Walter H. Eddy, Professor of Physiological Chemistry, Columbia University. Cloth. 93 pages. \$3.00. Williams & Wilkins Co., Baltimore, Md.

### Statistical Methods for Research Workers\*

THIS new edition of a well-known and deservedly popular book contains many changes and additions so that the book should be even more useful than before.

The relation of mathematics to the presentation and statistical analysis of research data is well recognized and even obvious. Not all engaged in research are sufficiently oriented in the methods of the statistician to apply them to their problems. To these, this book will be of inestimable value for it is clear, understandable, and practical.

### Drug Addicts Are Human Beings†

THIS is a book concerned with the narcotic problem.

Dr. Williams believes that the drug addict should be entitled to a freely available maintenance supply of the drug in question; that the Harrison Act is ineffective, works injustice to both physician and addict, that it places both in the power of the unscrupulous narcotic agent, and that, as a consequence, it has given way to a "billion dollar drug racket."

There is no question but that the handling of the narcotic problem leaves much to be desired. But this presentation of it and how it may best be solved would be much more effective if the style of this book were less reminiscent of De Kruif in his best and most hysterical days.

### Physical Diagnosis‡

PERHAPS few books on physical diagnosis have been more deservedly popular than Dr. Cabot's for few books of this character have been based on as comprehensive an experience or have presented the subject in a more practical and usable manner.

Those familiar with former editions—and their names are legion—will remember that Dr. Cabot's book was, in essence, a mirror of his own personal experience. Its aim was to express, as stated in the first preface, not merely what had been recommended, but rather what he, by actual experience, had found valuable.

This twelfth edition marks a departure in this respect, but not in the purpose or the character of the book as a whole. Realizing that no one individual experience, no matter how extensive, can embrace all that the modern study of physical diagnosis now comprises, Dr. Cabot has associated with him in the preparation of this new edition Dr. F. Dennette Adams as co-author.

This does not mean that "the personal equation" so characteristic of former editions is lost. On the contrary, it now presents the results of the personal experience, not only of the authors, but also of their colleagues in the Massachusetts General Hospital. The new edition, therefore, embraces even a wider field than its predecessors. It is larger in size and contains numerous entirely new illustrations.

The intensely practical character of the book is evident at a glance just as it is also evident that it represents practical and personal experience. It is without doubt a book of individuality, well organized, well written, well illustrated, and well indexed.

This book can be highly recommended without reserve.

\*Statistical Methods for Research Workers. By R. A. Fisher, Sc.D., F.R.S. Cloth, ed. 7. 356 pages, \$3.50. Oliver & Boyd, Edinburgh and London.

†Drug Addicts Are Human Beings. By Henry Smith Williams, M.D., B.Sc., LL.D. Cloth. 273 pages, \$2.50. Shaw Publishing Co., Washington, D. C.

‡Physical Diagnosis. By Richard C. Cabot, M.D., Professor of Clinical Medicine Emeritus; and F. Dennette Adams, M.D., Instructor in Medicine, Harvard Medical School Courses for Graduates. Cloth, ed. 12. 846 pages, 309 illustrations, \$5.00. William Wood & Co., Baltimore, Md.

# The Journal of Laboratory and Clinical Medicine

Vol 24

SEPTEMBER 1939

No 12

## CLINICAL AND EXPERIMENTAL

### THE INFLUENCE OF VITAMIN D ON THE SERUM PHOSPHATASE ACTIVITY IN ARTHRITIS\*

PAUL W. SMITH, PH D., A. DORF KLEIN, B S. AND IRVING E. STECK, M D  
CHICAGO, ILL.

IN RECENT years numerous reports have indicated that the phosphatase activity of the serum may vary widely from normal values, usually in an upward direction, in bone diseases of various types. The literature has been carefully reviewed by Kay,<sup>1</sup> and more recently by Morris and Peden.<sup>2</sup> On the other hand, relatively little attention has been paid to the possibility of phosphatase activity changes in chronic arthritis of the atrophic or hypertrophic types, although they may involve osseous changes of considerable magnitude. Kay<sup>3</sup> reported that plasma phosphatase activity values in arthritis may vary at random within, above, or below the accepted normal range. Rhee<sup>4</sup> did not report values significantly different from normal ones.

During the past few years we have had available for study a large number of cases of chronic arthritis from which all but the atrophic and hypertrophic types have been excluded by careful diagnosis. At the start of this investigation a large proportion of these patients had been taking some form of vitamin D in massive doses (150,000 to 400,000 units per day), with results comparable to those previously described.<sup>5</sup>

We were interested in finding whether the serum phosphatase activity differs in the two types of the disease and the possible effects upon it of prolonged administration of large amounts of vitamin D.

In all, 29 persons were studied divided into groups as follows. Group A, consisting of 18 atrophic and a subgroup of 5 hypertrophic patients who had

\*From the Departments of Physiology and Medicine, College of Medicine, University of Illinois.

A part of the expenses of this investigation was borne by a grant from the Nutrition Research Laboratories.

Received for publication January 25, 1939.

### Statistical Methods for Research Workers\*

THIS new edition of a well known and deservedly popular book contains many changes and additions so that the book should be even more useful than before.

The relation of mathematics to the presentation and statistical analysis of research data is well recognized and even obvious. Not all engaged in research are sufficiently oriented in the methods of the statistician to apply them to their problems. To these, this book will be of inestimable value for it is clear, understandable, and practical.

### Drug Addicts Are Human Beings†

THIS is a book concerned with the narcotic problem.

Dr. Williams believes that the drug addict should be entitled to a freely available maintenance supply of the drug in question; that the Harrison Act is ineffective, works injustice to both physician and addict, that it places both in the power of the unscrupulous narcotic agent, and that, as a consequence, it has given way to a "billion dollar drug racket."

There is no question but that the handling of the narcotic problem leaves much to be desired. But this presentation of it and how it may best be solved would be much more effective if the style of this book were less reminiscent of De Kruif in his best and most hysterical days.

### Physical Diagnosis‡

PERHAPS few books on physical diagnosis have been more deservedly popular than Dr. Cabot's for few books of this character have been based on as comprehensive an experience or have presented the subject in a more practical and usable manner.

Those familiar with former editions—and their names are legion—will remember that Dr. Cabot's book was, in essence, a mirror of his own personal experience. Its aim was to express, as stated in the first preface, not merely what had been recommended, but rather what he, by actual experience, had found valuable.

This twelfth edition marks a departure in this respect, but not in the purpose or the character of the book as a whole. Realizing that no one individual experience, no matter how extensive, can embrace all that the modern study of physical diagnosis now comprises, Dr. Cabot has associated with him in the preparation of this new edition Dr. F. Dennette Adams as co author.

This does not mean that "the personal equation" so characteristic of former editions is lost. On the contrary, it now presents the results of the personal experience, not only of the authors, but also of their colleagues in the Massachusetts General Hospital. The new edition, therefore, embraces even a wider field than its predecessors. It is larger in size and contains numerous entirely new illustrations.

The intensely practical character of the book is evident at a glance just as it is also evident that it represents practical and personal experience. It is without doubt a book of individuality, well organized, well written, well illustrated, and well indexed.

This book can be highly recommended without reserve.

\*Statistical Methods for Research Workers. By R. A. Fisher, Sc.D., F.R.S. Cloth, ed. 7, 356 pages, \$3.50. Oliver & Boyd, Edinburgh and London.

†Drug Addicts Are Human Beings. By Henry Smith Williams, M.D., B.Sc., LL.D. Cloth, 273 pages, \$2.50. Shaw Publishing Co., Washington, D.C.

‡Physical Diagnosis. By Richard C. Cabot, M.D., Professor of Clinical Medicine Emeritus, and F. Dennette Adams, M.D., Instructor in Medicine, Harvard Medical School Courses for Graduates. Cloth, ed. 12, 846 pages, 309 illustrations, \$5.00. William Wood & Co., Baltimore, Md.

# *The Journal of Laboratory and Clinical Medicine*

Vol 24

SEPTEMBER, 1939

No 12

## *CLINICAL AND EXPERIMENTAL*

### THE INFLUENCE OF VITAMIN D ON THE SERUM PHOSPHATASE ACTIVITY IN ARTHRITIS\*

PAUL W. SMITH, PH D., A. DRO KLEIN, B S. AND IRVING E. STECK, M D  
CHICAGO, ILL

IN RECENT years numerous reports have indicated that the phosphatase activity of the serum may vary widely from normal values, usually in an upward direction, in bone diseases of various types. The literature has been carefully reviewed by Kay,<sup>1</sup> and more recently by Morris and Peden.<sup>2</sup> On the other hand, relatively little attention has been paid to the possibility of phosphatase activity changes in chronic arthritis of the atrophic or hypertrophic types, although they may involve osseous changes of considerable magnitude. Kay<sup>3</sup> reported that plasma phosphatase activity values in arthritis may vary at random within, above, or below the accepted normal range. Race<sup>4</sup> did not report values significantly different from normal ones.

During the past few years we have had available for study a large number of cases of chronic arthritis from which all but the atrophic and hypertrophic types have been excluded by careful diagnosis. At the start of this investigation a large proportion of these patients had been taking some form of vitamin D in massive doses (150,000 to 400,000 units per day), with results comparable to those previously described.<sup>5</sup>

We were interested in finding whether the serum phosphatase activity differs in the two types of the disease, and the possible effects upon it of prolonged administration of large amounts of vitamin D.

In all, 29 persons were studied, divided into groups as follows. Group A, consisting of 18 atrophic and a subgroup of 5 hypertrophic patients who had

\*From the Departments of Physiology and Medicine, College of Medicine, University of Illinois.

A part of the expenses of this investigation was borne by a grant from the Nutrition Research Laboratories.

Received for publication January 25, 1939.



been receiving vitamin D prior to the first phosphatase observations, which were made at intervals of approximately one month with occasional omissions when patients did not report regularly. The observations extended over periods of three to nine months. Group B, 6 atrophic patients upon whom were made two phosphatase determinations, one week apart, after which viosterol administration was started. Observations then followed at irregular intervals of one week to one month, extending over five months while vitamin D administration continued.

In addition to the foregoing, a group of normal subjects, 19 in all, were studied, to establish a normal range of individual variations as well as a normal group range with the methods of analysis used.

TABLE I  
PHOSPHATASE ACTIVITY, BODANSKY UNITS, NORMAL YOUNG MALES

SUBJECT	AGE	BLOOD PRESSURE	FIRST MONTH	SECOND MONTH	THIRD MONTH	FOURTH MONTH	VARIATION GREATER THAN 1 UNIT	AVERAGES
R. C.	27	125/70	2.20	2.90	3.20		1.00	2.79
H. DeC.	49	210/120	4.00	3.25	3.82			3.69
P. K.	26	124/70	3.02	2.90	1.98		1.04	2.63
B. A.	23	119/74	2.01	3.42	1.98		1.44	2.47
W.	24	127/84	4.29	4.02	3.86			4.06
K.	22	123/75	2.54	2.21	2.76			2.50
R. L.	17	120/73	4.29	3.96	4.10			4.12
								(Highest)
L. B.	28	136/83	1.77	2.90	2.36	2.0	1.33	2.26
G.	21	121/78	4.20	3.84	3.60	4.19		3.96
D. R.	23	118/70	3.56	3.47	3.51	3.14		3.42
R. L.	22	110/90	2.92	3.54	3.54			3.33
D. K.	23	135/80	3.24	3.90	2.91	3.51		3.39
Y. T.	21	129/86	3.28	2.90	3.81	3.50		3.37
C.	20	126/78	4.41	3.81	4.21			4.11
C. M.	26	129/83	2.95	2.61	2.05			2.54
J. S.	22	123/78	1.98	3.64	2.76		1.66	2.79
B.	24	130/85	3.96	4.25	4.11			4.11
D. V.	22	126/90	2.01	1.90	1.90			1.94
								(Lowest)
H. C.	24	120/78	3.52	2.89	0.40		1.11	3.47
							Average	3.21

All phosphatase determinations were done by the method of Bodansky,<sup>6</sup> with the modification that serum inorganic phosphate was determined in a mixture containing serum, buffer, and substrate, in every way identical with the hydrolysis mixture except that trichloroacetic acid was added immediately after addition of the substrate. Because of the interference of both the substrate (sodium beta glycerophosphate) and the trichloroacetic acid with the development of color in the Kuttner-Lichtenstein method for inorganic phosphate (Bodansky<sup>7</sup>), the values for serum inorganic phosphate are low to the extent of about 10 per cent. Since the inorganic phosphate liberated by hydrolysis is obtained by the difference between this first determination and one after two hours' incubation at constant temperature, the unit values for phosphatase activity are reasonably accurate without correction. For the phosphate analyses we used a photoelectric colorimeter with a suitable spectral filter. Since the instrument is calibrated with phosphate standards

over a wide range of concentrations, deviations from the Beer's law do not introduce an error.

There was no possibility of maintaining either the experimental or normal subjects on a standard diet, and dietary variations may possibly have contributed to the variability of phosphatase activity observed in individuals from month to month, since Freeman and Farmer<sup>8</sup> demonstrated that values may increase on a high carbohydrate diet and decrease as a result of a high protein diet. Venous blood samples were taken routinely in the afternoon shortly after lunch. Bodansky and Jaffe<sup>7</sup> indicated that there is no detectable difference between samples taken immediately after a meal and after a brief fast.

In Table I are given the phosphatase values for a control group of 19 adult males. With one exception (a man of 49, average blood pressure 210/120) all were between the ages of 17 and 28 and gave normal average blood pressure readings, which facts are partial evidence of good general health.

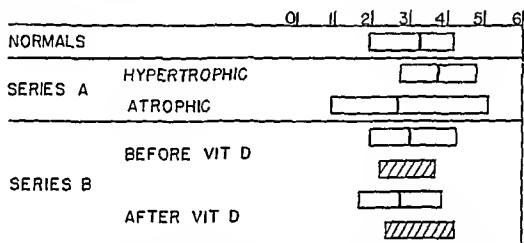


Fig. 1.—Distribution of the phosphatase activity of all experiments. Scale represents Bodansky units; heavy lines dividing rectangles averages; cross hatched rectangles series B serum inorganic phosphate. Scale represents mg phosphorus per 100 cc of serum.

The highest phosphatase activity encountered was 4.41 units and the lowest 1.90. In 6 of the 19 subjects or 32 per cent monthly variations exceeded 1 unit, the greatest variation being 1.66 units. As shown in the last column, the averages of the three or four determinations on a single subject ranged from 1.94 units to 4.12 units, with a general average of 3.21. All of these fall within, or reasonably close to, the normal range of 1.5 to 4.0 units, as defined by Cantarow.<sup>10</sup>

In contrast with these results observe the spread of values in Table II, in which are summarized the values for group A, arthritic patients receiving vitamin D. The lowest value encountered was 0.44 units, patient E B, and the highest 5.40, patient F J. The individual averages revealed a spread of values from 0.92 to 5.08 units. The general average for the 18 atrophic patients was 2.69 units, and for the 5 hypertrophic patients 3.67. In 14 of the 23 atrophic and hypertrophic patients (61 per cent) the variations from month to month exceeded 1 unit, the greatest being 2.65 units in patient B B.

These group averages and the spread of values are graphically presented in Fig. 1, which also includes the comparable data for the normal control subjects.

TABLE II  
SERUM PHOSPHATASE ACTIVITY, BODANSKY UNITS, AFTER PROLONGED ADMINISTRATION OF VITAMIN D. ATROPHIC AND HYPERTROPHIC ARTHRITIS

SUBJECT	AGE WT.	SEX	FIRST DET.	FIRST MO.	SECOND MO.	THIRD MO.	FOURTH MO.	FIFTH MO.	SIXTH MO.	SEVENTH MO.	NINTH MO.	AVERAGE PHOS- PHATASE	VARIA- TION GREATER THAN 1 UNIT	TREND PHOSPHATASE
<i>Atrophic Series</i>														
E. M.	$\frac{36}{140}$	F	2.03	2.40	2.10	2.17	2.21		1.87	2.18		2.14		Little change
E. C.	$\frac{30}{130}$	F	2.28	2.66		1.80	1.58	1.67	2.21			2.03	1.08	Random
V. C.	$\frac{20}{90}$	F	2.40	2.76	2.62					2.22		2.50		Little change
N. S.	$\frac{29}{140}$	F	2.99	2.39	3.57	3.64		2.49	3.16			3.21	1.15	Random
E. B.	$\frac{24}{120}$	F	1.18	1.36		0.68			0.44			0.92		Downward
G. T.	$\frac{49}{130}$	F	4.29	3.62		2.67	3.67					3.56	1.62	Random
L. K.		F	2.36	2.13	1.05	2.13						1.92	1.31	Random
P.	$\frac{26}{126}$	F	1.98	1.67	1.71							1.79		Little change
B. B.	$\frac{59}{159}$	F	3.72		1.99	2.49		4.64				3.21	2.65	Random
A. L.	$\frac{26}{127}$	M	3.04	2.93	1.36	2.46	2.99					2.56	1.68	Random
W. M.	$\frac{52}{165}$	M	3.72	2.72	2.75	2.21	2.58	2.75				2.79	1.52	Random'

TABLE II—Cont D

[illegible]

There is no apparent correlation between the phosphatase activity and the clinical picture in this group of patients, as indicated by the following brief protocols, representing patients who received varying degrees of relief as the result of vitamin D therapy.

*Patient:* E. M.

*Date of Admission:* July, 1936.

*Complaining of:* Pain, stiffness, swelling and deformity of hands, left knee, ankles, and feet (seven years' duration).

*Findings to Date:* March 3, 1938. The swelling was much decreased; pain was present only when grasping objects. She had no pain in hands when washing dishes, although the stiffness was about the same, and she was still unable to close her fingers tightly. The flexion of the left knee was markedly improved; she was able to extend knee fully, and there was very little swelling. There was no pain in knee on resting or walking. Very little swelling in the left ankle, although the right ankle was still swollen, and both ankles gave pain when patient walked or stood for any length of time. There was no improvement in the ankles as far as pain was concerned, and both feet were still painful.

*Summary.*—There was improvement in the hands and the left knee, but no improvement in the ankles and feet. First phosphatase determination was made on Feb. 2, 1937. Serum phosphatase showed fair constancy, slightly above 2 units.

*Treatment:* The patient received 150,000 units of vitamin D (ertron) daily, with period of therapeutic vacation.

*Patient:* E. C.

*Diagnosis:* Atrophic arthritis.

*Date of Admission:* October 1, 1937.

*Complaining of:* (1) Pain, swelling, stiffness and limited motion of both elbows; (2) pain, swelling, stiffness and limited motion of both wrists; (3) pain, swelling, stiffness and limited motion of hands and fingers; unable to close fingers and had a very weak grip; (4) pain, swelling, stiffness and flexion deformity in both knees; (5) pain, swelling, stiffness and limited motion in both ankles; (6) pain and swelling in neck and jaw (four years' duration).

*Findings to Date:* March 17, 1938: When patient was first admitted, she complained of severe pain in both elbows on pressure, and was unable to move elbows on account of pain. She had no pain at this observation in either elbow on ordinary motion, and only slight pain on pressure. She was able to comb her hair, and could lift a pail of water with no pain whatsoever in either elbow. The pain, swelling, and stiffness in both wrists were much decreased, and there was a slight increase in motion of wrists; she also had greater power in both wrists. She could now wring out towels with no discomfort, while on admittance she was unable to perform these tasks. The swelling and flexion deformity of hands and fingers were greatly decreased and she was able to close fingers completely, with a very good grip, whereas on admission she could not use a pencil to write on account of this severe pain in fingers. The pain, swelling, and stiffness of both knees were much less; she was able to walk more freely and to straighten out knees more readily. She walked 8 blocks to and from work without any discomfort; she was unable to do this when admitted. She had greater mobility in ankles; swelling and pain were much decreased, with only occasional pain in ankles when walking.

*Summary:* On admittance to the clinic this patient was tired and weak, the slightest exertion exhausted her. She had gained about 20 pounds and was stronger and did not tire so easily. She was unable to hold a job on admittance, but had been employed since Jan. 5, 1938, and worked eight hours a day, made her own meal on coming home, and was able to do this without any strain. On admittance she had pain all the time whether resting or in mo-

tion. She had pain only occasionally on walking, at the final observation. First phosphatase determination was made on Feb. 2, 1937. Serum phosphatase varied at random with tendency toward a decrease over the total period of observation.

*Treatment* The patient received 180,000 units of vitamin D (viosterol), treatment continued.

*Patient A. L.*

*Diagnosis* Atrophic arthritis.

*Date of Admission* Jan. 14, 1937.

*Complaining of* Pain, swelling and stiffness in all joints (two months' duration).

*Findings to Date* April, 1938. On admission patient could not dress or feed himself on account of severe pain, swelling and stiffness in both shoulders, elbows, wrists, hands, and fingers. He came to the clinic in a wheelchair, he could not walk on account of the pain in the ankles and knees.

*Summary* At final observation this patient was working eight hours a day, was able to dress and feed himself, could walk without pain or any discomfort. First phosphatase determination was made on Jan. 14, 1937. A decline of phosphatase activity in early months of vitamin D therapy was followed by a rise, similar to patients of series B, Table III.

*Treatment* 150,000 units of vitamin D (ertion) discontinued in October, 1937.

*Patient W. G.*

*Diagnosis* Atrophic arthritis.

*Age* Thirty-five years.

*Date of Admission* November, 1936.

*Complaining of* Flexion deformity of the right elbow, pain, swelling, and stiffness of both wrists, both hands, and all fingers, flexion deformity of left knee and right ankle. Gradual onset (four years' duration).

*Findings to Date* April 13, 1938. On admission the patient could not dress or feed himself, both hands were weak and painful. He could not lift light objects. At present he can do all of these without difficulty. The stiffness and swelling of both hands were improved. The swelling, stiffness, and pain in the left knee were completely absent, and he could walk long distances without any pain, whereas on admission to the clinic he could walk only two blocks and then had to rest on account of pain in left knee and right ankle. The flexion deformity had improved to the extent that he could extend the knee to about 165°.

*Summary* The patient was much stronger, he could walk with less difficulty. His wrists, hands, and fingers were stronger, although the stiffness and swelling were still present, pain was absent, except on exertion. The patient had gained 18 pounds. First phosphatase determination was made on Jan. 28, 1937. Phosphatase varied at random over a range of 118 units.

*Treatment* This patient received 150,000 to 200,000 units of vitamin D daily, he is still under treatment.

*Patient F. J.*

*Date of Admission* July, 1936.

*Complaining of* The patient complained of pain in the back of neck, pain on motion of both shoulders, pain and flexion deformity of both elbows, with "olecranon bursitis" in both elbows. He also complained of pain, stiffness, swelling and limited motion of hands and fingers, pain and stiffness of both knees, pain, stiffness, and swelling of both ankles, and severe pain on the soles and heels of both feet (three years' duration).

*Findings to Date* March 3, 1938. The pain in the back of neck had completely disappeared. There was only slight pain in the shoulders and he could move shoulder joints without feeling any pain. He had no pain in the elbows, however, the deformity had not improved. The motion was still limited, but he could move elbows without any pain. The

swelling in the hands varied. He was able to use his hands without pain, whereas on admission he could not hold a cup in his hand. He now had a good grip and could work, i.e., hold a hammer firmly, etc. He was able to close fingers almost fully at times. He had no pain in knees, only slight pain on arising in the morning, which disappeared within fifteen minutes. He had no more pain in either ankle, although the swelling was still prominent. The heels and soles of both feet were painless, and he was able to walk long distances without pain or discomfort.

*Summary:* This patient was badly crippled and unable to walk when he was admitted to the clinic. In fact, at times he had to miss visits to the clinic because he was not able to walk to the street car. He could not feed himself because of severe pain in hands and fingers. At final observation he could do manual work without discomfort, and he could walk long distances without any pain. He claimed he felt much stronger generally. First phosphatase determination was made on Feb. 4, 1937; phosphatase was very high. His condition remained quite stable around 5 units.

*Treatment:* The patient received 150,000 to 250,000 units of vitamin D (ertron) daily and is still under treatment at 200,000 units per day.

TABLE III

SERUM PHOSPHATASE ACTIVITY IN ATROPHIC ARTHRITIS. VITAMIN D ADMINISTRATION BEGUN AFTER SECOND PHOSPHATASE DETERMINATION

SUBJECT	SEX	FIRST DET.	MONTHS AFTER FIRST DETERMINATION				AVERAGE PHOS- PHATASE BEFORE	AVERAGE PHOS- PHATASE AFTER
			SECOND	THIRD	FOURTH	FIFTH		
T. U	F	2.23 2.12* 1.66	1.39		1.88		2.18	1.64
A. M.	F	3.96 4.50* 3.53	3.18	4.05			4.23	3.59
E.	F	3.23 2.22* 3.29	2.71		2.03	1.50	2.83	2.38
J. M.	F	2.09 1.83* 1.99	1.37	1.58	2.18	1.62	1.96	1.75
E. G.	F	3.56 3.49* 4.65	3.00	3.58 4.24			3.53	3.82
R.	F	2.82 3.42* 1.88	2.94 3.07 5.20 2.95	2.41 3.66			3.12	3.10
Group average							2.98	2.71

\*Beginning of vitamin D administration.

Table III summarizes the results of frequent phosphatase activity determinations on the 6 atrophic patients of group B. The results show that the phosphatase activity has been depressed by the vitamin. This is demonstrated graphically in Fig. 1; along with the uncorrected serum inorganic phosphate values, which show a reciprocal shift, there is a tendency for the phosphatase activity to fall sharply immediately after the beginning of medication, with a tendency toward a later rise. A few typical protocols are included to show

that improvement in the clinical picture seems to correspond with the magnitude of the difference between premedication and postmedication averages. The group is small, however, and we do not feel it safe to press this point.

*Patient T U*

*Diagnosis* Atrophic arthritis

*Date of Admission* March 11, 1937

*Complaining of* (1) Pain, stiffness, and flexion deformity of both elbows, (2) pain, swelling, stiffness, and limited motion of both wrists, (3) both hands and all fingers painful and swollen, (4) pain, swelling, and stiffness in both knees, (5) pain, swelling, and stiffness in both ankles (three years' duration).

*Findings to Date* March 17, 1938. There was less stiffness and flexion deformity in elbows and the patient could extend both elbows more easily than on admission. She had very little pain in either elbow, and she could use her wrists more freely. The swelling on the right wrist had decreased, but she had a huge ganglion over the left wrist. She complained of no pain in hands or fingers and could close fingers, whereas on admission she could not do this due to the severe pain. She had a good strong grip. The swelling in both knees had decreased. On admission she had severe pain in knees either on resting or walking. She was now able to walk 1 mile or more without pain in either knee. The pain and swelling in both ankles were definitely decreased and there was no pain in ankles when walking.

*Summary* The general condition of this patient had improved greatly. She was weak and the slightest exertion exhausted her when she first came to the clinic. She gained about 5 pounds. She was not able to walk when first she came to the clinic, but she was now able to do light housework with no discomfort.

*Treatment* This patient received 150,000 to 300,000 units of vitamin D (ertron) since admittance. She is still under treatment.

*Patient A M*

*Date of Admission* April 1937

*Complaining of* Pain, swelling, and crepitation of both knees.

*Comparative Findings* April 14, 1938. On admission the patient had severe and continuous pain in both knees. She was unable to sleep and she could not walk on account of pain. At the latter date she was able to walk 8 or 9 blocks with very slight pain. Crepitation was still present in knees, but no swelling.

*Summary* On admission this patient could hardly walk. A year later she could walk with very little difficulty. Crepitation was still present but there was no swelling.

*Treatment* This patient received 150,000 units vitamin D (ertron) with no periods of vacation. She is still under treatment.

*Patient J M*

*Age* Fifty three years

*Diagnosis* Atrophic arthritis

*Date of Admission* April 15, 1937

*Complaining of* (1) Pain and flexion deformity of both elbows, (2) pain, swelling, and limited motion of both wrists and both hands, (3) pain, swelling, and stiffness of all fingers, (4) pain and swelling in both knees, (5) pain in both feet and all toes (eight years' duration).

*Findings to Date* March 10, 1938. The patient still complained of pain and flexion deformity in both elbows, and pain and swelling in both wrists, also of pain and slight swelling in her hands and fingers, less pain in knees, feet and toes. She was able to walk more freely.



*Summary:* There was no improvement in the elbows, wrists, and hands, but there is less pain in knees, feet, and toes.

*Treatment:* The patient received 150,000 to 250,000 units of vitamin D (ertron) daily with periods of therapeutic vacation. She is under treatment.

*Patient:* P. R.

*Diagnosis:* Atrophic arthritis.

*Date of Admission:* May, 1936.

*Complaining of:* Pain in shoulders, elbows, wrists, fingers, knees, and ankles (two years' duration).

*Comparative Findings:* July 21, 1938. Patient still complained of severe pain in all joints.

*Summary:* Condition showed no improvement.

*Treatment:* This patient received 150,000 to 400,000 units of vitamin D and is still under treatment at the clinic.

#### DISCUSSION AND SUMMARY

Those patients who were examined for serum phosphatase activity after prolonged medication with vitamin D have, in general, a lower serum phosphatase activity than do healthy normal subjects. That this is an effect of the vitamin in large doses there is little doubt. Page and Reside<sup>11</sup> observed lowered phosphatase activity after large doses of vitamin D, attributing the fall to toxic impurities in the preparation. Crum and Strayer,<sup>12</sup> however, believed that the lowered phosphatase value is a manifestation of the toxicity of high dosage with vitamin D, independent of impurities. There is no evidence that the arthritic state in itself is associated with a lowering of phosphatase activity; indeed, in those cases investigated before medication both high and low values were encountered, and in the large series summarized in Table II, there were many values above normal, especially in the hypertrophic cases, despite prolonged vitamin D administration. The serial determinations on the individuals in this study who had been receiving vitamin D over a long period of time show either a random variation about the average value or a downward trend. The tendency for the values to rise after an initial fall in the B group of patients might be ascribed to a developing tolerance to the toxic effects of the drug, after which a plateau is reached, this being the approximate status of the A group.

The fact that no apparent correlation exists between the absolute value of the phosphatase activity in units and the degree of clinical improvement in the A series (Table II) is perhaps to be expected, since Table III (Group B) indicates the possibility of a correlation between degrees of improvement and magnitude of the shift from premedication values, but not between improvement and absolute phosphatase values.

In this regard it should be profitable to study the phosphatase activity in arthritis under other types of treatment to ascertain whether the improvement obtained in a high percentage of cases as the result of vitamin D therapy is a specific response to the reduction in phosphatase activity which vitamin D causes in susceptible patients, or whether improvement and reduction in phosphatase activity are merely coincidental.

## CONCLUSIONS

It is concluded from this study, first, that serum phosphatase activity does not promise to be of value as an objective diagnostic aid in arthritis due to the wide distribution of values above, below, and within the normal range. The possibility of its value as an aid in differentiating between the atrophic and hypertrophic types is again negated by the wide distribution, regardless of the somewhat higher average obtained by us on a limited number of hypertrophic cases.

Second, there is a possibility that the shift in the serum phosphatase activity, which we have observed to occur in patients who have ultimately benefited from vitamin D, might prove to constitute a basis for prediction as to the suitability of such therapy. Further investigation is needed on this point.

## REFERENCES

- 1 Kay, H. D. Phosphatase in Growth and Disease of Bone. *Physiol Rev* 12: 384, 1932.
- 2 Morris, N., and Peden, O. D. Plasma Phosphatase in Disease. A Review, *Quart J Med* 6: 211, 1937.
- 3 Kay, H. D. Plasma Phosphatase. II. The Enzyme in Disease. Particularly in Bone Disease, *J Biol Chem* 89: 249, 1930.
- 4 Rice, J. The Phosphatase Test in the Arthritides and Osteitides, *Arch M Hyg* 10: 6, 1932.
- 5 Dreyer, I., and Reed, C. I. Treatment of Arthritis With Massive Doses of Vitamin D, *Arch Phys Therapy* 16: 537, 1935.
- 6 Bodansky, A. Determination of Plasma Phosphatase. *Proc Soc Exper Biol & Med* 28: 760, 1931.
- 7 Bodansky, A., Hallman, L., and Bonoff, R. Phosphatase Studies. I. Determination of Inorganic Phosphate, Beer's Law and Interfering Substances in the Kuttner-Lichtenstein Method, *J Biol Chem* 99: 197, 1932-1933.
- 8 Freeman, S., and Farmer, C. I. Correlated Studies of Calcium Inorganic Phosphorus, and Serum Phosphatase in Animals Influenced by Ergosterol, *Am J Physiol* 113: 209, 1935.
- 9 Bodansky, A., and Jaffe, H. L. Phosphatase Studies. III. Serum Phosphatase in Diseases of the Bone. Interpretation and Significance. *Arch Int Med* 54: 88, 1934.
- 10 Cantarow, A. Review of Phosphatase Activity and Calcium Electrolyte Metabolism. *Internat Clin* 1: 230, 1936.
- 11 Page, I. H., and Reside, D. M. Die Wirkung von bestrahltem Ergosterin auf Gewebe Phosphatase, *Biochem Ztschr* 223: 171, 1930.
- 12 Crumm, P. D., and Straver, J. W. Phosphatase Content of Blood Serum and Tissues in the Rat Following Administration of Vitamins D and A. *J Biol Chem* 112: 511, 1936.

# THE NEURAL DEPRESSING EFFECT OF TRICHLORETHYLENE\*

H. S. RUBINSTEIN, PH.D., M.D., E. PAINTER, PH.D., AND O. G. HARNE  
BALTIMORE, MD.

TRICHLORETHYLENE has been used with a fair degree of success in the treatment of trigeminal neuralgia (Plessner, 1916; Glaser, 1931). The beneficial effect derived in these cases was considered to be due to the specific depressant action which this chemical exerted upon the trigeminal nerve. For this reason, and because of the preponderant innervation of the dura by the trigeminal nerve (Foerster, 1926), this same drug was used in a series of migraine patients with encouraging results (Rubinstein, 1937).

However, because examination of such treated patients failed to disclose a limited trigeminal anesthesia which observation substantiated the findings of others (Goldberg, 1924; Bieder, 1921), it became questionable whether trichlorethylene did produce its effect through a limited specific action. The following study was therefore undertaken.

A series of dogs was anesthetized with ether and the dura mater and right sciatic nerve of each were exposed. Each animal was then allowed to come to a very light anesthetic stage. Observations were then made in which the dura mater and sciatic nerve were stimulated with a faradic current. Since the dura is free from nerve endings in many of its areas, exploratory stimulation was carried out until a response was obtained (see Fig. 1). This selected point was then used for subsequent stimulation. The response was measured as deviations in blood pressure obtained through a recording manometer inserted into the carotid artery.

Following a series of responses observed under deep and light ether anesthesia, the animal was allowed to inhale 2 to 3 c.c. of trichlorethylene administered through a tracheal cannula. The animal soon went into a deep sleep during which time stimulation of the dura or sciatic failed to produce any reflex effect upon blood pressure. As sleep wore off the dura and sciatic nerves were again stimulated with faradic current and observations were again made. Fifty-two such observations were made before and 52 after the use of trichlorethylene.

All data obtained were tabulated and treated by the usual statistical methods (Pearl, 1930). Means for each series of observations were then compared with their respective controls and ratios of significance were determined. Ratios of 3 or more were considered to be significant.

\*From the Research Laboratory, Department of Physiology, College of Physicians and Surgeons, Mount Sinai Hospital, Baltimore; Department of Physiology, School of Medicine, Johns Hopkins University, Baltimore; and Department of Physiology, School of Medicine, University of Maryland, Baltimore.

Received for publication, January 12, 1939.